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AURINTRICARBOXYLIC ACID INHIBITION OF mRNA:RIBOSOME BINDING AND PHENYLALANYL-tRNA SYNTHETASE REACTIONS

BY

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A THESIS

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ABSTRACT

A study of the inhibitory action of ATA on two different steps of protein synthesis—the mRNA binding reaction and the phenylalanyl-tRNA synthetase reaction—was undertaken.

Studies of ATA action on the mRNA binding reaction revealed that ATA does not dissociate a preformed mRNA:ribosome complex. However, when added prior to the complex formation, ATA inhibits mRNA binding to 50% at a concentration of 20 μM. It was also confirmed that ATA inhibition is due to its binding to ribosomes. However, it was not possible to deduce the exact site and nature of ATA binding due to the complexity of ribosomal structure and due to the presence of protein factors closely associated with the ribosomes. An examination of the inhibition from the viewpoint of ATA structure employing various aurin derivatives showed that analogs of ATA are not very effective inhibitors of mRNA binding. It appeared that ATA is a unique inhibitor of mRNA binding due to the presence of hydroxyl and/or carboxyl groups on the triphenylmethane skeletal structure. During the course of the above studies, it was noted that there are other reactions sensitive to ATA besides the mRNA binding reaction, since the overall process of poly-U directed phenylalanine incorporation is inhibited at a lower concentration of ATA than the mRNA binding step. The search for a reaction sensitive to ATA led us to discover that the phenylalanyl-tRNA synthetase reaction is inhibited by ATA. The ATA inhibition of any aminoacyl-tRNA synthetase had not previously been reported. Thus, this novel system was analyzed. Studies with aurin derivatives showed that the analogs of ATA were all potent inhibitors of this reaction, indicating a different mode of ATA action than in the mRNA binding reaction. In spite of this

difference, the presence of hydroxyl and/or carboxyl groups enhances the inhibitory activity as was the case in ATA action on the mRNA: ribosome interaction. Studies into the site of ATA action in the synthetase reaction indicated that it binds to the enzyme . The stoichiometry of ATA binding to the synthetase was calculated to be approximately 6 ATA molecules per enzyme molecule. This binding of ATA to the synthtase was found to result in non-competetive inhibition of the binding of tRNA to the enzyme.

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LIST OF ABBREVIATIONS

AMP	adenosine 5'-monophosphate
CMP	cytidine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CTP	cytidine 5'-triphosphate
GTP	guanosine 5'-triphosphate
poly-U	polyuridylate
RNA	ribonucleate
mRNA	messenger ribonucleate
tRNA	transfer ribonucleate
s.v.tRNA	transfer ribonucleate treated with snake venom phosphodiesterase as described in (2-7)
Phe-tRNA	phenylalanyl-transfer ribonucleate
Phe	phenylalanine
ATA	aurintricarboxylic acid
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
DEAE	diethylaminoethyl
E. coli	Escherichia coli
cpm	counts per minute
A_n	absorbance at n nm
M	molar
mM	millimolar
μM	micromolar
mmoles	millimoles
μmoles	micromoles
nmoles	nanomoles
pmoles	picomoles

LIST OF ABBREVIATIONS (continued)

g	gram
mg	milligram
μg	microgram
ml	milliliter
μl	microliter
mm	millimeter
nm	nanometer
min	minute
°C	degrees Centigrade
MW	molecular weight
I_{50}	concentration of inhibitor sufficient to cause 50% inhibition of a reaction
G	gravitational force
S	Svedberg unit

CHAPTER 1

Introduction

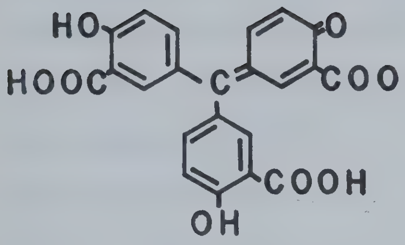
The protein synthesizing machinery of living systems is composed of a number of protein and RNA components which interact with each other in a specific manner. These components are: more than 40 kinds of tRNA specific to individual aminoacids, aminoacyl-tRNA synthetases which specifically recognize one of 20 amino acids, messenger RNAs whose numbers could be equal to the entire gene contents of an organism, protein factors which control each step of the protein synthesizing machinery (the three initiation factors, three translocation factors, and one or two termination factors), and ribosomes. The ribosome plays a central role in the protein synthesis by providing the binding sites for these reaction components (Haselkorn and Rothman-Denes 1973). The ribosome itself, however, is composed of two subunits, and in fact each subunit is made of RNA (28S and 5S for the larger subunit and 18S for the small subunit) and more than two dozen proteins. The complexity of this ribosomal structure and the specific recognition by protein subunits of a specific site of ribosomal RNA still remain a mystery even in the bacterial system (see Review by Kurland 1972). In fact, the mechanism of the specific protein:RNA interactions involved in protein synthesis is not elucidated to date.

In this regard, the Adaptor Hypothesis by Crick (1958) is perhaps the clearest illustration of the questions that must be answered to understand the process of protein synthesis. The original problem of how the nucleotide sequence is translated into an amino acid sequence is now being replaced by the new question of how to couple the amino acids to specific adaptors (tRNAs). After all, the enzymes, aminoacyl-

tRNA synthetases, are a sequence of amino acids, and they must recognize the specific combination or sequence of nucleotides of the tRNAs. Therefore, the elucidation of the mechanism controlling these protein:RNA interactions is extremely valuable in understanding protein synthesis. This study deals with an inhibitor of such protein:RNA interactions involved in an early stage of protein synthesis. In particular, it was our intention to study the action of aurintricarboxylic acid (ATA) and its derivatives on the mRNA:ribosome interaction and on the aminoacyl-tRNA synthetase reaction (see Fig. 1 for structure of ATA derivatives).

It was in 1951 that the aurin dyes first drew some attention in biomedical studies when White _et_ _al_ demonstrated that ATA can be used in the treatment of beryllium poisoning in mice. Their studies into the mechanism of ATA action indicated that ATA likely removes beryllium from its site of action by chelating the ion (Schubert _et_ _al_ 1952, Lindenbaum _et_ _al_ 1954, Lindenbaum and White 1954). This mechanism appears to analogous to the technique of mordanting cotton in dye technology. The test using triphenylmethane compounds other than ATA failed in the poison treatment (White and Schubert 1954, Lindenbaum and White 1954). Later studies on the distribution of ATA in the tissue of beryllium-poisoned mice led to the conclusion that the ATA:Be complex bound to protein fibers of the tissue examined (Lindenbaum _et_ _al_ 1954, Schubert and Lindenbaum 1954, Lindenbaum and Lisco 1956).

At about the same time, physical and chemical studies on the dye:protein interaction were progressing with emphasis on developing probes into conformational aspects of the proteins (Oster 1951, Brown 1951, Shimao 1952, Kusunoki 1952). Brown studied the decoloration of dye solutions by proteins, and speculated that the decoloration of dye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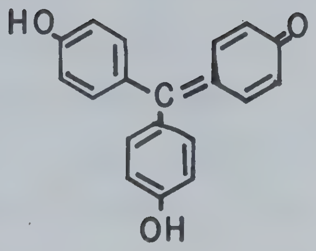
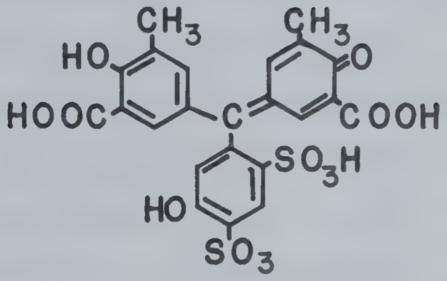
HOOC
C
O
COO
COOH
OH

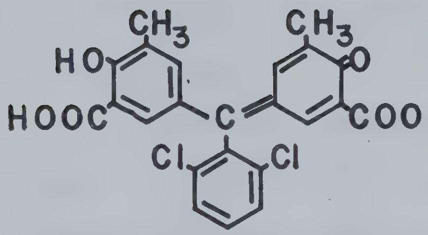
ATA



AURIN


CH3
CH3
HO
O
HOOC
C
COOH
SO3H
HO
SO3

ERICHROME CYANINE R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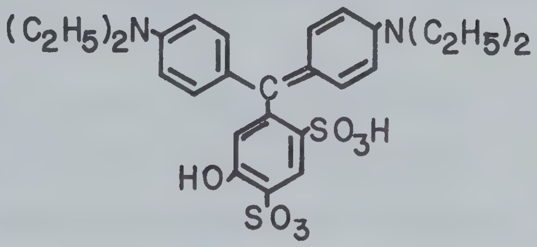
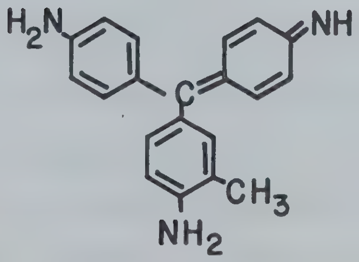
CH3
HO
O
HOOC
C
COO
Cl
Cl

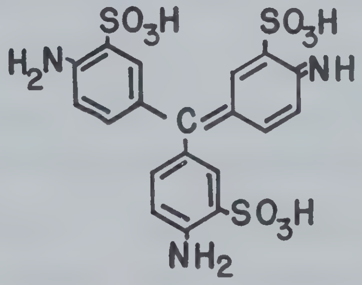
AZURE BLUE B


(C2H5)2N
N(C2H5)2
C
SO3H
HO
SO3

PATENT BLUE


H2N
NH
C
CH3
NH2

FUCHSIN BASIC


SO3H
SO3H
H2N
NH
C
SO3H
NH2

FUCHSIN ACID

Fig. 1 Structures of the aurin derivatives used in the mRNA binding reaction and in the phenylalanyl-tRNA synthetase reaction.

solutions may be due to complex formation between the dye and the added protein molecules. It was also found that the oxidation of SH-groups results in reduced decoloration of dye by protein, indicating the involvement of SH-groups in the dye:protein interaction. Brown further postulated that the site of interaction on the dye molecule was the central carbon atom of the triphenylmethane dye.

It was almost two decades after the work of Brown's group that the scientific value of ATA was rediscovered. Grollman and Stewart (1968) reported that ATA inhibits the translation of bacteriophage f2 RNA in the *E. coli* cell-free system. They demonstrated that ATA action is exerted at the initiation step of protein synthesis. This was a novel finding since there had been no inhibitor specific to the initiation step at that time. Thus the role of ATA in arresting the reaction at the mRNA:ribosome interaction is of considerable interest in the field of protein synthesis.

Immediately following the report by Grollman and Stewart (1968), the effect of ATA on a variety of nucleotidyl transferase reactions and on protein biosynthesis was examined by the members of Dr. Igarashi's laboratory—including myself. The enzymes tested were: DNA-dependent RNA polymerase of *E. coli*, R17 RNA-dependent RNA polymerase, tRNA 3'-terminal nucleotidyl transferase from *E. coli* and L cells, 16 aminoacyl-tRNA synthetases and synthetic mRNA binding to ribosomes (all unpublished data). All of the enzymic reactions tested were found to be inhibited by ATA. This broad spectrum of enzymes susceptible to ATA suggested to us that ATA is not a specific inhibitor, contrary to the initial proposal by Grollman and Stewart (1968). However,all of the reactions tested appear to be sensitive rather specifically to ATA.

Other triphenylmethanes tested require concentrations 10 to 100 times that of ATA to exert the same level of inhibition. We thus directed out study toward the elucidation of the mechanism by which ATA uniquely inhibits the enzyme reaction in hope of providing some insight into the specific recognition of RNA by protein. The present study was therefore aimed specifically at the determination of which components of the mRNA binding reaction and of the aminoacyl-tRNA synthetase reaction, both of which occur in the initial stages of protein synthesis, could complex with ATA. An attempt was also made to determine the effect of ATA on the binding of other ligands to the enzyme.

This study employed the incorporation of phenylalanine in the reticulocyte cell-free system as directed by poly-U. At the outset, the concentration of ATA required for 50% inhibition of the mRNA binding reaction was established. Further studies showed that the site of ATA action is on the ribosome, but that the preformed mRNA:ribosome complex is resistant to ATA action. The determination of the ATA binding site was not possible due to the complexity of the ribosome structure and the presence of associated proteins. An examination of the inhibition from the viewpoint of ATA structure was carried out using ATA analogs. The results showed that the presence of carboxyl and/or hydroxyl groups is required for inhibitory action.

For the examination of ATA action on aminoacyl-tRNA synthesis, the Phe-tRNA synthetase reaction was used. The study using ATA analogs in this system suggested a different mode of action from that in the mRNA binding reaction, even though the carboxyl and/or hydroxyl groups are necessary for the inhibition. The results clearly show that ATA binds to the enzyme and quantitation of the ATA:enzyme interaction

indicated that there is a specific number of ATA molecules bound per enzyme molecule, at approximately a ratio of 6:1. An examination of the effect of ATA on the binding of other ligands to the enzyme showed that it interferes with the binding in a non-competative manner.

CHAPTER 2

MATERIALS AND METHODS

(2-1) Radioactively Labelled Chemicals

The following radioactively labelled chemicals, with specific activities as given in parenthesis, were used in this study. ^{3}H-CTP (20.4 Ci/mmole) and ^{3}H-phenylalanine (5-16 Ci/mmole) were purchased from New England Nuclear. ^{3}H-polyuridylate (52.8 mCi/mmole polynucleotide phosphorus) was from Miles Laboratories Inc.

(2-2) Chemicals

ATP,GTP, and CTP were from Schwarz/Mann. Polyuridylate was purchased from Miles Lab. Inc. Phosphoenolpyruvate, Trizma base, heparin, and phenylhydrazine-HCl were from Sigma. Puromycin was from Nutritional Biochemicals. All of the triphenylmethane dyes except ATA (a Baker product) were obtained from Harleco. Sepharose, Sephadex, and Agarose were purchased from Pharmacia Fine Chemical Co. DEAE-cellulose was from Carl Schleicher and Schuell Co. PPO (2,5-diphenyloxazole) and POPOP (1,4-bis(2-(5-phenyloxyzoylbenzene)), which were used in scintillation fluid, were purchased from New England Nuclear. All other chemicals were reagent grade from Baker.

(2-3) Biological Materials

New Zealand white rabbits (2-3 kg) were used for the preparation of reticulocyte cells. Yeast tRNA (unfractionated) and phosphodiesterase from *Crotalus adamanthus* venom were purchased from Calbio-

chem. The marker proteins (glucose oxidase, chymotrypsinogen A, and lysozyme) used for molecular weight estimation were obtained from Sigma.

(2-4) Buffered Solutions

The following buffered solutions were used wherever suitable: Buffer A (Tris 10 mM, KCl 5 mM, $MgCl_2$ 0.2 mM, sucrose 50 mM, final pH 7.6). Buffer B (Tris 50 mM, KCl 5 mM, $MgCl_2$ 0.2 mM, final pH 7.8). Buffer RM (Tris 100 mM, KCl 60 mM, $MgCl_2$ 10 mM, β-mercaptoethanol 4 mM, final pH 7.8). Buffer RN (Tris 25 mM, KCl 40 mM, $MgCl_2$ 5 mM, β-mercaptoethanol 5 mM, final pH 6.5).

(2-5) Preparation of Rabbit Reticulcyte Polysomes and Monosomes

Reticulocyte cells were obtained from New Zealand white rabbits (2-3 kg) which had been treated with five daily injections of 2.5% neutralized phenylhydrazine HCl at a dosage of 0.33 ml per kilogram body weight per injection (Burny and Marbaix 1964). After a two-day resting period, the rabbits were anesthetized by injection of 2 ml Nembutal (50 mg/ml) into the marginal vein of the ear. Immediately, heparin (2 ml of a 1% solution) was injected. Then blood was collected through the marginal vein of the ear using a vacuum apparatus. The percentage of reticulocytes was examined under a microscope. When the reticulocytes exceeded 85% of the total blood cells, the blood was processed as follows: the cells were lysed in a hypotonic saline solution and insoluable materials were removed by centrifugation at 10,000 x G. The resulting supernatant was further clarified by centrifugation at 30,000 x G. This fraction was then subjected to high speed centrifugation at 142,000 x G for 40 min in a preparative ultracentrifuge to precipitate polysomes. The resulting supernatant was then incubated at

37° for 40 min in the presence of added puromycin (1 mM) and GTP (0.1 mM) in order to convert residual polysomes to monosomes. The monosomes were precipitated at 142,000 x G for 90 min and suspended in Buffer A. The monosome concentration was adjusted to 100 A_{260} units per ml and stored in a liquid nitrogen refrigerator.

(2-6) Preparation of Aminoacyl-tRNA Synthetase from Reticulocyte Cells

A mixture of aminoacyl-tRNA synthetases was prepared from the supernatant of the high speed centrifugation, post-monosomal supernatant, as described in section (2-5). Two volumes of saturated ammonium sulfate in Buffer A were added to one volume of the supernatant, and the resulting solution was allowed to stand in the cold for 60 min. The precipitate was then collected by centrifugation at 15,000 x G for 30 min in a Beckman model J21 refrigerated centrifuge. The precipitate was dissolved in Buffer A to give a protein concentration of 20 mg/ml, and was stored at -20°C until use. This fraction was designated as the AS66-fraction.

Partial purification of Phe-tRNA synthetase was performed as follows: a portion of AS66 fraction was dialyzed overnight against Buffer B at 4°C in order to reduce the concentration of the monovalent cation to approximately 20 mM KCl equivalent. The reason for the choice of Buffer B is that the higher Tris concentration is required to maintain constant pH during the subsequent column chromatography. The dialyzed fraction was fractionated on a DEAE cellulose column using a batchwise method. The column was first developed with Buffer B and then with Buffer B containing 0.1 M ammonium sulfate. Phe-tRNA synthetase activity was found in this 0.1 M ammonium sulfate fraction, while the

majority of hemoglobin was eluted in the Buffer B wash. The fractions containing the synthetase activity were pooled and dialyzed at 4°C for 18 hr against Buffer B containing saturated ammonium sulfate. The precipitate formed in the dialysis tubing was collected by centrifugation at 15,000 x G. The precipitate was then dissolved in a minimal volume of Buffer B. This enzyme fraction was then subjected to molecular seiving chromatography using Sepharose 6B. A 20 mg portion of the enzyme fraction was loaded onto a Sepharose 6B column of bed volume 150 ml and processed with Buffer B containing 150 mM ammonium sulfate. Each fraction was examined for Phe-tRNA synthetase activity , and active fractions were pooled and dialyzed against saturated ammonium sulfate in Buffer B overnight. The precipitate was collected by centrifugation, and dissolved in Buffer B to give a final concentration of 10 mg/ml. This fractionation procedure achieved a 50-fold increase in specific activity of the synthetase from the AS66 fraction.

(2-7) Preparation of Snake Venom Phosphodiesterase Treated tRNA (s.v.tRNA)

In order to provide the substrate for the tritiation of tRNA, yeast tRNA was subjected to a limited digestion of the 3'-terminal end by snake venom phosphodiesterase. An incubation mixture contained in 2 ml, tRNA 50 mg, phosphodiesterase 0.045 units, lysine 0.1 M (pH 9), $MgCl_2$ 1.2 mM. After 30 min reaction at 37°C, the reaction mixture was extracted three times with 1 ml of water saturated phenol. From the final aqueous layer, RNA was precipitated by the salt-methanol method of Igarashi and McCalla (1971). The precipitate was washed with ether-methanol, dried, and dissolved in water.The tRNA treated with snake venom phosphodiesterase under the conditions specified

lacks the 3'-terminal residue -CA and in small proportions -CCA and is designated as s.v.tRNA (Igarashi and McCalla 1971).

(2-8) Radioactivity Measurement

The filters that retained radioactive materials were placed in vials containing 5 ml of the scintillation fluid composed of PPO 6 g, POPOP 0.6 g, in toluene 1 liter. The radioactivity was measured in a Beckman Scintillation System LS-200B. A 10% counting efficiency was obtained for tritium.

(2-9) Preparation of ^{3}H-yeast tRNA

The method used to prepare labelled yeast tRNA involved the addition of ^{3}H-CMP to the 3'-terminal of s.v.tRNA catalized by 3'-terminal nucleotidyl transferase of L-cells (Igarashi and Larratt 1974). The reaction mixture (0.1 ml) contained lysine (pH 8.5) 100 mM, $MgCl_2$ 1 mM, $MnCl_2$ 1 mM, KCl 200 mM, β-mercaptoethanol 0.5 mM. In addition, 10 μCi of ^{3}H-CTP, 1 mg of s.v.tRNA and 100 μg of tRNA 3'-terminal nucleotidyl transferase were added. This reaction mixture was incubated at 37°C for 60 min. At 60 min, 0.1 nmoles of CTP per 0.1 ml of reaction mixture was added, incubated at 37°C for 10 min and finally 0.5 μmoles ATP per 0.1 ml reaction mixture was added and incubated an additional 20 min at 37°C. To the reaction mixture, an equal volume of water saturated phenol was added. After 5 min of vigorous mixing using a Vortex mixer, the aqueous layer was separated from the phenol layer by means of centrifugation at 15,000 x G for 5 min at 15°C. In order to ensure clear separation, the rotor was stopped by coasting. The aqueous layer was re-extracted with phenol. Then two volumes of methanol were added to pre-

cipitate tRNA. After one hour of standing in the cold, the precipitate was collected by centrifugation at 10,000 x G for 10 min. The pellet was washed twice with a salt-methanol mixture (1 M KCl : methanol = 3:7, in volume), once with a methanol-ether mixture (1:1) and finally with ether. The final pellet was dissolved in distilled water to a RNA concentration of 10 mg/ml, yeilding 17,000 cpm per microliter.

(2-10) Assay Method for Polyphenylalanine Synthesis

Protein synthesis in the reticulocyte cell-free system was measured by following the poly-U directed incorporation of ^{3}H-phenylalanine into a hot TCA insoluable material. The reaction mixture (0.1 ml) contained: Tris (pH 7.8) 100 mM, $MgCl_2$ 10 mM, KCl 20 mM, β-mercaptoethanol 5 mM, ATP 1 mM, GTP 5 μM, phosphoenolpyruvate 0.5 mM. In addition, 2 μCi of ^{3}H-phenylalanine, 10 μg poly-U, 100 μg of post-ribosomal proteins and 2 A_{260} units of ribosomes were added. The reaction took place at 37°C for 60 min. At intervals, 0.1 ml aliquots were withdrawn and placed on filter discs. The discs were processed with the hot TCA wash method, and then subjected to radioactivity measurement as previously described (Igarashi and Paranchych 1967). In short, the filter discs were soaked in cold 10% TCA and allowed to stand in the cold for 40 min, followed by 5% TCA wash twice at room temperature. Discs were then heated to 90°C for 45 min in 5% TCA, and washed twice with 5% TCA. They were extracted with ether-ethanol (1:1) mixture for 15 min and finally with ether for 10 min. After being completely dried, the discs were placed in a toluene scintillation fluid. The radioactivity retained on each filter was measured as described in (2-8).

(2-11) Assay Method for Phe-tRNA Synthesis

Aminoacyl-tRNA synthetase activity was measured by the incoporation of ^{3}H-phenylalanine into a cold TCA insoluable fraction. The reaction mixture (0.1 ml) contained: Tris (pH 7.8) 100 mM, $MgCl_2$ 10 mM, KCl 30 mM, β-mercaptoethanol 5 mM, and ATP 1 mM. In addition, 2 μCi of ^{3}H-phenylalanine, 100 μg of tRNA and 100 μg of the synthetase were added to each 0.1 ml of reaction mixture. The reaction took place at 37°C for 20 min. At intervals, 0.1 ml aliquots were removed and processed with the cold TCA wash (Igarashi and Paranchych 1967). In short, the discs were immersed in cold 10% TCA for 40 min, followed by four successive washes with cold 5% TCA for 15 min each time. They were then extracted with ether-ethanol (1:1) for 15 min, and finally with ether for 10 min. After being dried, the discs were placed in a toluene scintillation fluid and subjected to radioactivity measurement as described in (2-8).

(2-12) Assay Method for Poly-U Binding to Ribosomes

^{3}H-poly-U binding to ribosomes was measured either by the membrane filtration method using Millipore filters DA (pore size 0.65μ) (Igarashi and Paranchych 1967), or by the gel-filtration method using Agarose A50M. In both methods, ^{3}H-poly-U (20 μmoles polynucleotide phosphorus) was mixed with ribosomes (5 A_{260} units) in 0.5 ml of the reaction mixture containing: Tris (pH 7.8) 50 mM, $MgCl_2$ 5 mM, KCl 40 mM, and β-mercaptoethanol 1 mM. The reaction mixture was incubated in the cold (4°C) for 5 min, and examined for mRNA binding by one of the two methods mentioned above.

For the Millipore filtration method, the samples were diluted

with 5 ml of Buffer RM and filtered through the filters using a suction apparatus. The membrane filters were further washed with 10 ml of Buffer RM containing bovine serum albumin (1 mg/ml). After being dried, each filter was examined for radioactivity retained as described in (2-8). A control sample, which did not contain ribosomes, was processed similarly. The difference in radioactivities between experimental and control samples represented the amount of ^{3}H-poly-U bound to ribosomes. It should be mentioned that these membrane filters had to be pretreated in order to reduce nonspecific retention of ^{3}H-poly-U by soaking the filters in 0.1 N NaOH, rinsing with distilled water several times, and then soaking in 0.1 N HCl. Finally, they were washed with distilled water until neutralized, dried at room temperature, and tested for nonspecific retention of the polynucleate by random sampling. This pretreatment of filters reduced the background of ^{3}H-poly-U retention to a negligible level (less than 200 cpm).

For the gel-exclusion method, an Agarose A50M column of bed volume 10 ml was used. The reaction mixture (0.5 ml) was loaded onto the column, and then developed with Buffer A. In order to determine the elution position of ribosomes as well as of poly-U, they were processed separately on the same column under identical elution conditions to those above. Since the elution positions of these two components are quite far apart when processed separately, the radioactivity which eluted at the same position as the ribosomes was attributed to poly-U binding to ribosomes.

(2-13) Assay Method for ^{3}H-tRNA Binding to Phe-tRNA Synthetase

^{3}H-tRNA binding to the synthetase was measured using Millipore filters DA. The partially purified synthetase (50 μg) was mixed with

^{3}H-tRNA (10 pmoles) in 0.5 ml of the reactiom mixture containing: Tris (pH 6.5) 25 mM, $MgCl_2$ 5 mM, KCl 40 mM, and β-mercaptoethanol 5 mM. The reaction mixture was incubated in the cold for 5 min, diluted with 5 ml of Buffer RN, and filtered through the membrane filters using a suction apparatus. The filters were then washed with 10 ml of Buffer RN containing 1 mg/ml of bovine serum albumin, and examined for radioactivity as described in (2-8).

(2-14) Sepharose 6B Column Chromatography

Sepharose 6B column chromatography was used for two types of experiments under identical conditions. The first of these was designed to detect ATA binding to individual reaction components. The second was to estimate the molecular weight of the enzyme. For both studies, a column of bed volume 5 ml (5 mm x 130 mm) was used and developed with Buffer RM. For the use of such a small column, the volume of sample was limited to 50 μl.

For the binding studies, the reaction mixture was chromatographed first, followed immediately by chromatography of individual reaction components. In order to determine the peak positions of these components, the effluent was monitored at suitable wavelengths: 260 nm for tRNA, 280 nm for proteins and 308 nm for ATA.

For the molecular weight determination, the enzyme solution was first processed on the column. Then four proteins of known molecular weight were chromatographed in order to obtain the correlation between the elution volume vs. molecular weight. These marker proteins used were: glucose oxidase, 2.86×10^5; hemoglobin, 6.8×10^4; chymotrypsinogen A, 2.5×10^4; and lysozyme, 1.4×10^4.

CHAPTER 3

THE EFFECT OF ATA AND AURIN DERIVATIVES ON mRNA:RIBOSOME BINDING

(3-1) Introduction

The initiation of polypeptide synthesis in biological systems involves a series of reactions leading to the formation of the complex designated as the 'initiation' complex (Nomura and Lowry 1967) which includes the ribosome, mRNA, and aminoacyl-tRNA as the three key components. In addition, a number of protein factors control the interactions among these three reaction components (Revel and Gross 1966). Although the general features of the process leading to the formation of this initiation complex are now well understood, the nature of the interaction between the individual reaction components has not yet been elucidated. One of the reasons for such a delay in understanding the mechanism may be attributed to the lack of suitable inhibitors for the individual steps in complex formation. For this reason, the report that ATA was a specific inhibitor of the initiation of polypeptide synthesis (Grollman and Stewart 1968) was a significant discovery. This finding was made using the bacterial cell free system and soon was confirmed by other groups using mammalian and plant cell-free systems (Lebleu _et al_ 1970, Marcus _et al_ 1970). With the hope that a detailed analysis of ATA inhibition of initiation complex formation could shed some light on the mechanism of interaction between mRNA and ribosomes, we decided to undertake a study of the effect of ATA on the binding of poly-U to rabbit reticulocyte ribosomes.

The studies described in this chapter involve the determina-

tion of the inhibitory concentration of ATA in polyphenylalanine synthesis and in the poly-U:ribosome binding reactions, the determination of the site of ATA action, and the determination of the structural features of ATA responsible for the observed inhibition. These studies led us to conclude that ATA is the most effective inhibitor among the aurin derivatives tested, and that ATA forms a stable complex with ribosomes. ATA thus appears to be a unique inhibitor of mRNA:ribosome interaction. However, as will be described in Chapter 4, it was found that ATA analogs inhibit reactions other than the mRNA:ribosome complex formation, in particular the synthesis of the precursor molecule--aminoacyl-tRNA. Thus,ATA is not a specific inhibitor for mRNA:ribosome interaction (Igarashi and Zmean 1974).

(3-2) Results

Effect of ATA on Polyphenylalanine Synthesis

The inhibitory concentration of ATA in the poly-U directed polyphenylalanine synthesis was determined using rabbit reticulocyte monosomes supplemented by the AS66 fraction as a source of Phe-tRNA synthetase and other factors. The complete reaction mixture as described in the Methods was used with the presence of varying concentrations of ATA. The reaction was carried out at 37°C for 40 min and the samples were processed as described in the Methods. The level of ^{3}H-phenylalanine incorporation at 40 min of reaction was expressed as percent of the control experiment which did not contain ATA. Fig. 2 demonstrates that ATA inhibits the overall protein synthesizing activity to 50% at approximately 5 μM (I_{50} = 5 μM). Thus the inhibition of reticulocyte protein synthesis was established.

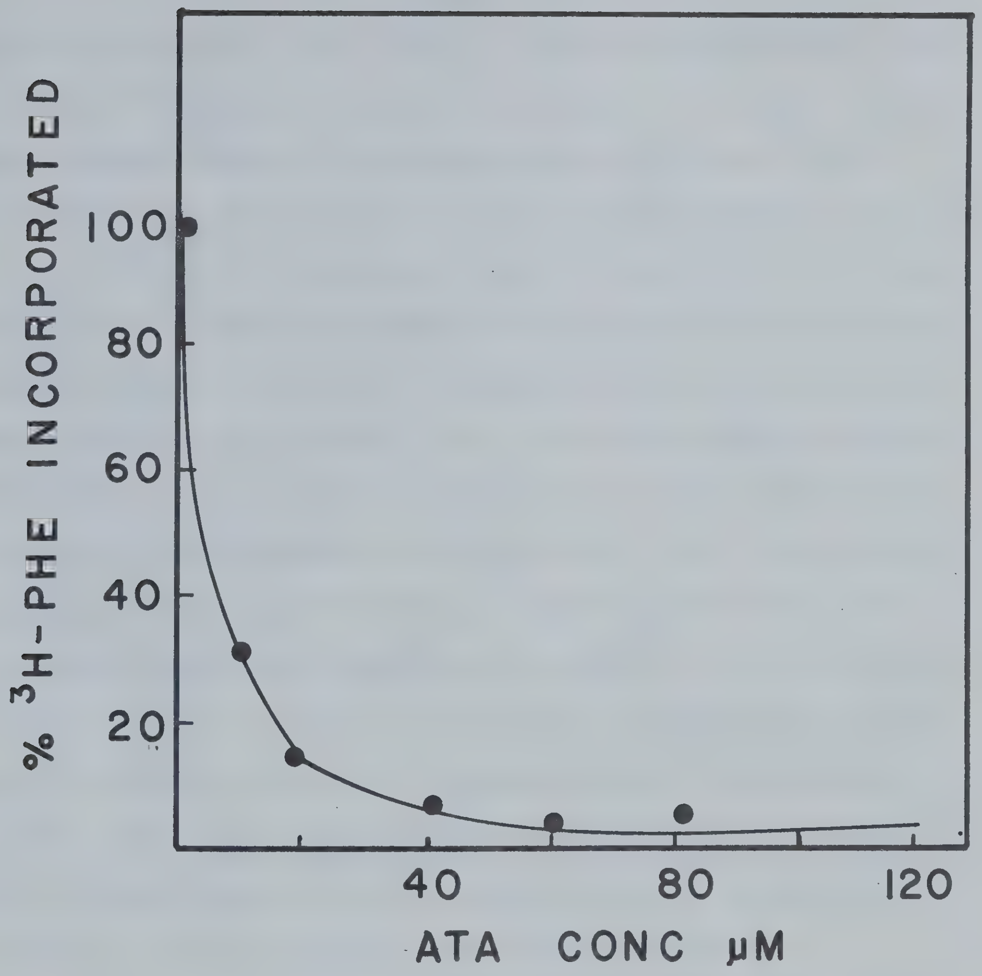


Fig. 2 Inhibition of polyphenylalanine synthesis by ATA. Poly-U directed ^{3}H-Phe incorporation was assayed as described in the Methods (2-10). The incorporation of ^{3}H-phenylalanine in the absence of ATA was taken as 100%. Activity demonstrated in the presence of varying amounts of ATA was expressed as a percent of the control value.

Effect of ATA on the mRNA Binding to Monomeric Ribosomes

At the time this investigation was undertaken, it was generally believed that ATA was a specific inhibitor of mRNA:ribosome complex formation. Therefore, an experiment was designed to determine whether the foregoing inhibition of polyphenylalanine synthesis by ATA is attributable solely to an inhibition of the poly-U:ribosome interaction. In order to do so, the interaction between monomeric rabbit reticulocyte ribosomes and ^{3}H-poly-U was examined in two ways. The first of these methods involved the use of the membrane filtration method employing Millipore filters DA (pore size 0.65μ). The reaction mixture containing ribosomes (5 A_{260} units) and ^{3}H-poly-U (20 nmoles polynucleotide phosphorus) was incubated in cold Buffer RM for 5 min, then subjected to membrane filtration. The effect of various concentrations of ATA on the binding reaction was examined and expressed by percent inhibition. As shown in Fig. 3 the level of ATA required for 50% inhibition (I_{50}) of the reaction is 20 μM. It is noteworthy that this I_{50} value is slightly higher than the I_{50} for overall polyphenylalanine synthesis. This difference led us to the investigation of other ATA sensitive reactions in protein synthesis as described in Chapter 4.

The second method used to study the effect of ATA on the binding of mRNA to ribosomes was the gel exclusion method using Agarose A50M. The reaction conditions had to be slightly modified from those used above due to the limited sensitivity of this method. The ratio of ATA to ribosomes was set at I_0 (no ATA, 5 A_{260} units of ribosomes), I_{50} (20 μM ATA, 5 A_{260} units of ribosomes), and I_{80} (40 μM ATA, 5 A_{260} units of ribosomes). An excess of poly-U was used to ensure maximum binding of poly-U to ribosomes in the system. Although the column

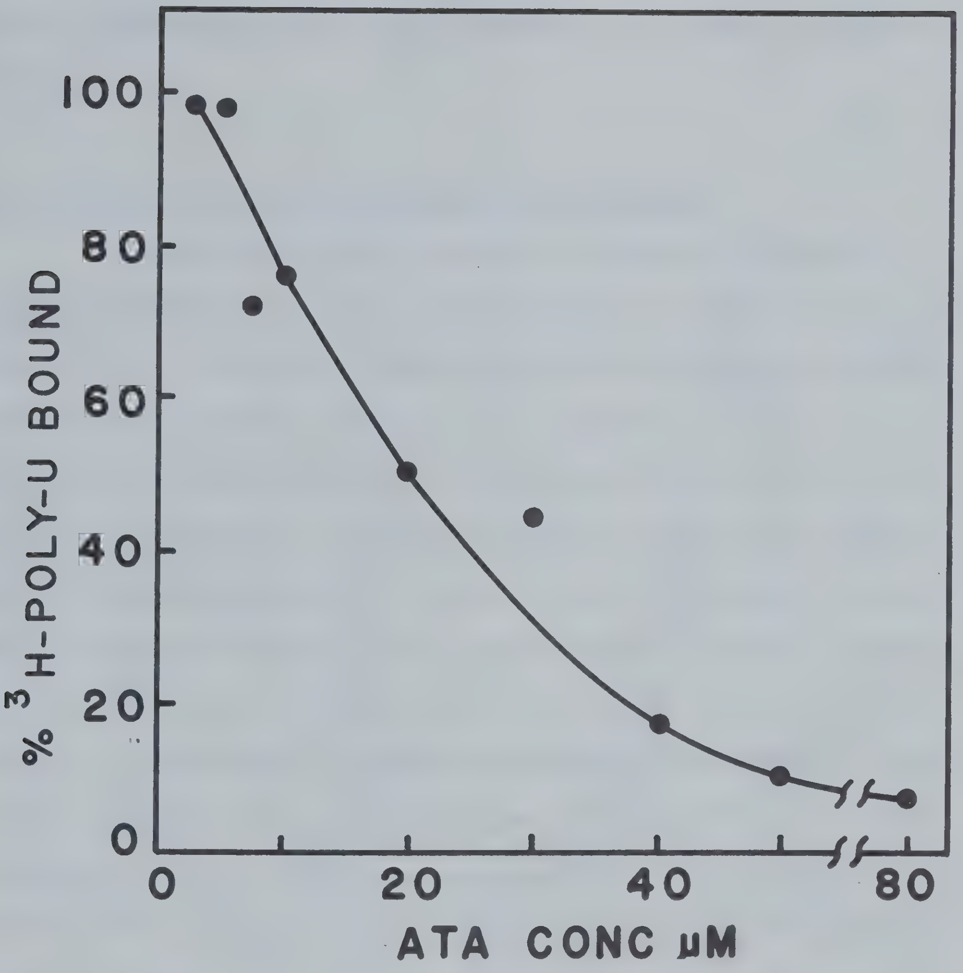


Fig. 3 ATA inhibition of ^{3}H-poly-U binding to monomeric ribosomes. Assays were performed as given in the Methods using Millipore filtration. The reaction mixture contained 5 A_{260} units of ribosomes, 20 nmoles poly-U phosphorus, and various concentrations of ATA. The level of ^{3}H-poly-U bound to monomeric ribosomes in the absence of ATA was taken as 100% activity. ^{3}H-poly-U binding in the presence of varying amounts of ATA was examined, and the results were expressed as a percent of the control level. 100% corresponds to 18,000 cpm.

chromatography method gives rise to qualitive rather than quantitative data, Fig. 4 shows the expected inhibition values, and can be considered as a reconfirmation of the data obtained by the membrane filtration method.

Site of Action in the mRNA:Ribosome Binding Reaction

The next step in this study was to acertain which reaction component interacts with ATA. If ATA inhibition is mediated by complexing with one of the reaction components, then increasing the concentration of that particular component in the presence of a fixed concentration of the dye should relieve its inhibitory action. The first component to be examined in this regard, therefore, was poly-U. It was added in increasing amounts to a reaction mixture containing constant amounts of ATA and ribosomes. Fig. 5 shows that for all poly-U concentrations tested, the inhibition due to ATA was maintained at a level of about 60%. The effect of varying the ribosome concentration in this system was examined next, and the results are shown in Fig. 6. It may be seen that as the ribosome concentration was increased, there was a decrease in the inhibition of poly-U binding. The foregoing two experiments thus suggest that the ribosome most probably is the component which interacts with ATA. However, the results in Fig. 6 could also be interpreted in terms of ATA binding to impurities in the ribosome solution.

To exclude the latter possibility, a study was carried out whereby the isolation of a ribosome:ATA complex was achieved by gel exclusion chromatography. First, poly-U and ribosomes were individually processed through a Sephadex G25 column of 10 ml bed volume, using

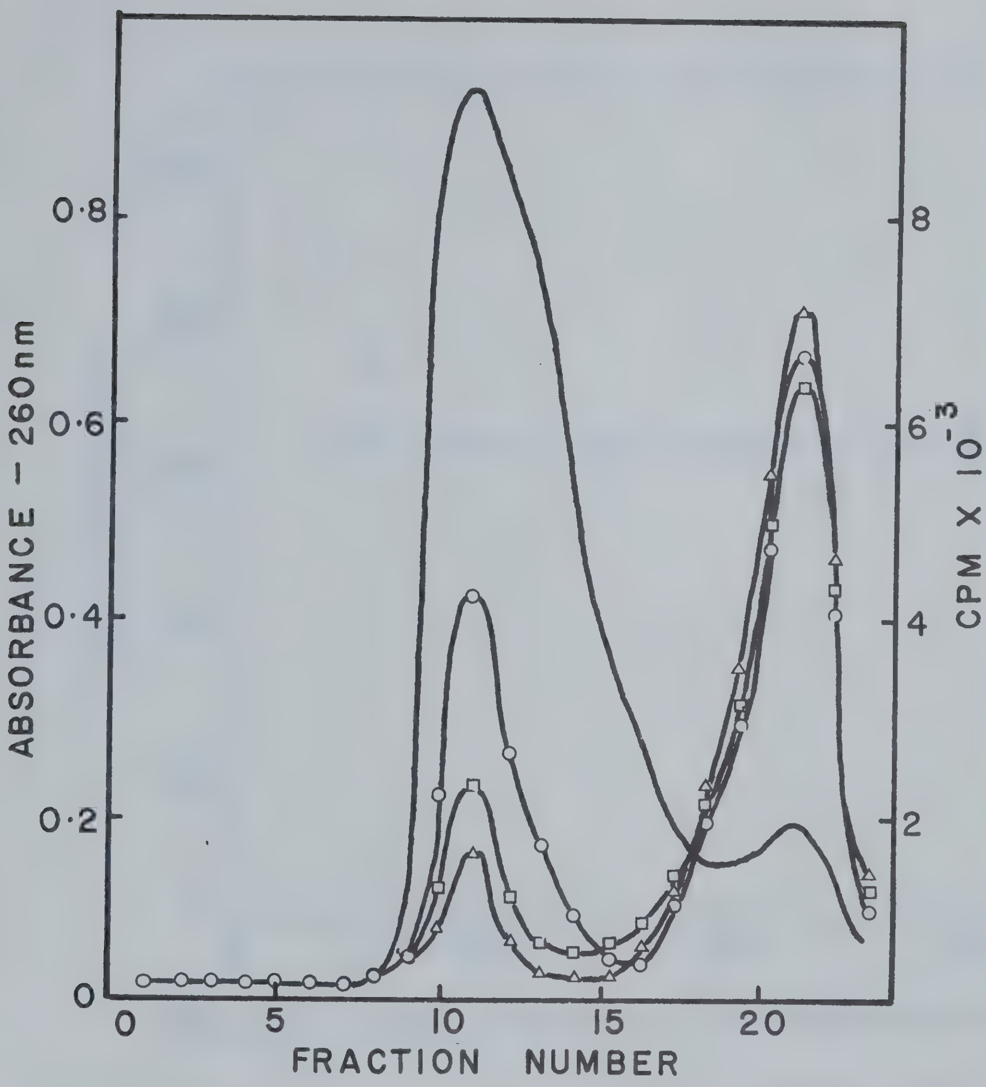


Fig. 4 ATA inhibition of ^{3}H-poly-U binding to monomeric ribosomes as detected by the gel exclusion method. Assays were performed as given in the Methods using Agarose A50M column chromatography. Ribosomes (20 A_{260} units) were mixed with excess ^{3}H-poly-U (200 nmoles phosphorus) in the presence of no ATA (○), 80 μM ATA (□) and 160 μM ATA (△). The mixtures were incubated in cold Buffer RM for 5 min, diluted with the same buffer and chromatographed. 260 nm absorption (——) and radioactivity for each fraction were measured.

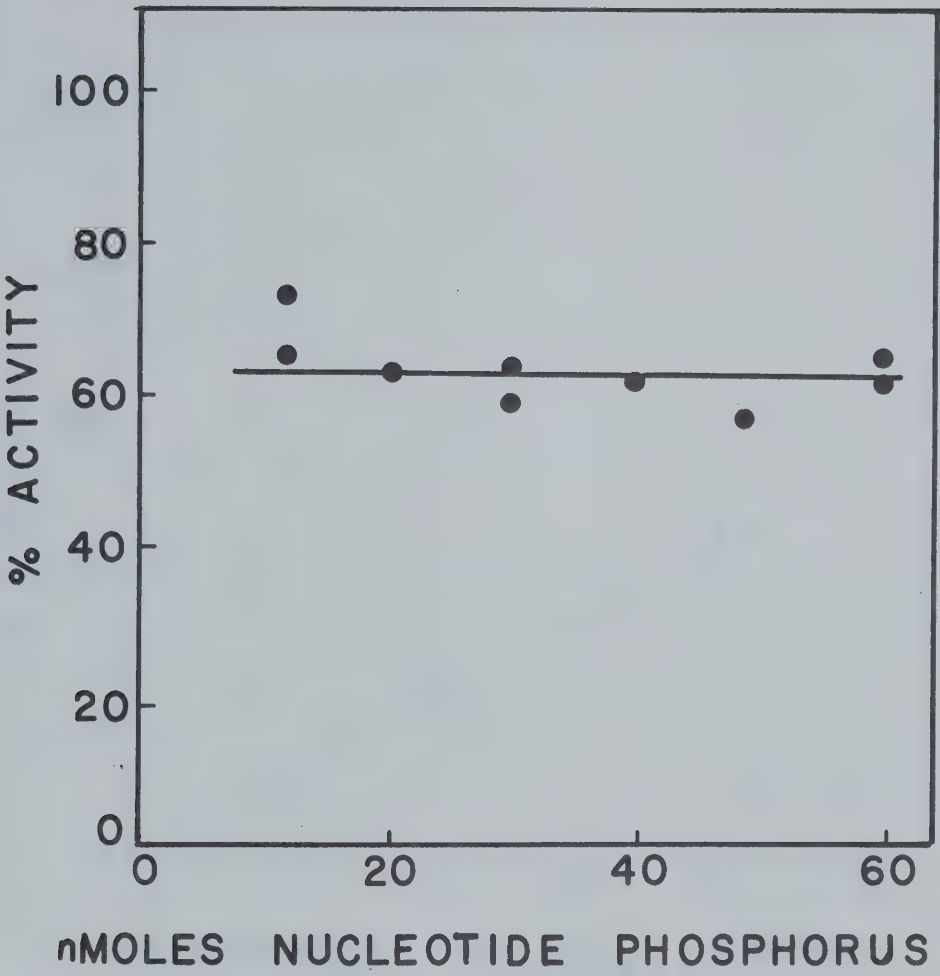


Fig. 5 Effect of ATA on poly-U:ribosome binding at various concentrations of poly-U. The reaction mixture (see Materials and Methods) contained 20 μM ATA, 5 A_{260} units of ribosomes, and ^{3}H-poly-U as indicated on the abscissa. The mRNA:ribosome complex retained on Millipore filters was estimated by measuring the radioactivity derived from ^{3}H-poly-U bound. Percent poly-U bound (ordinate) indicates the calculation based on the radioactivity in the experimental set over the value in the control set (no ATA present) at a certain concentration of poly-U.

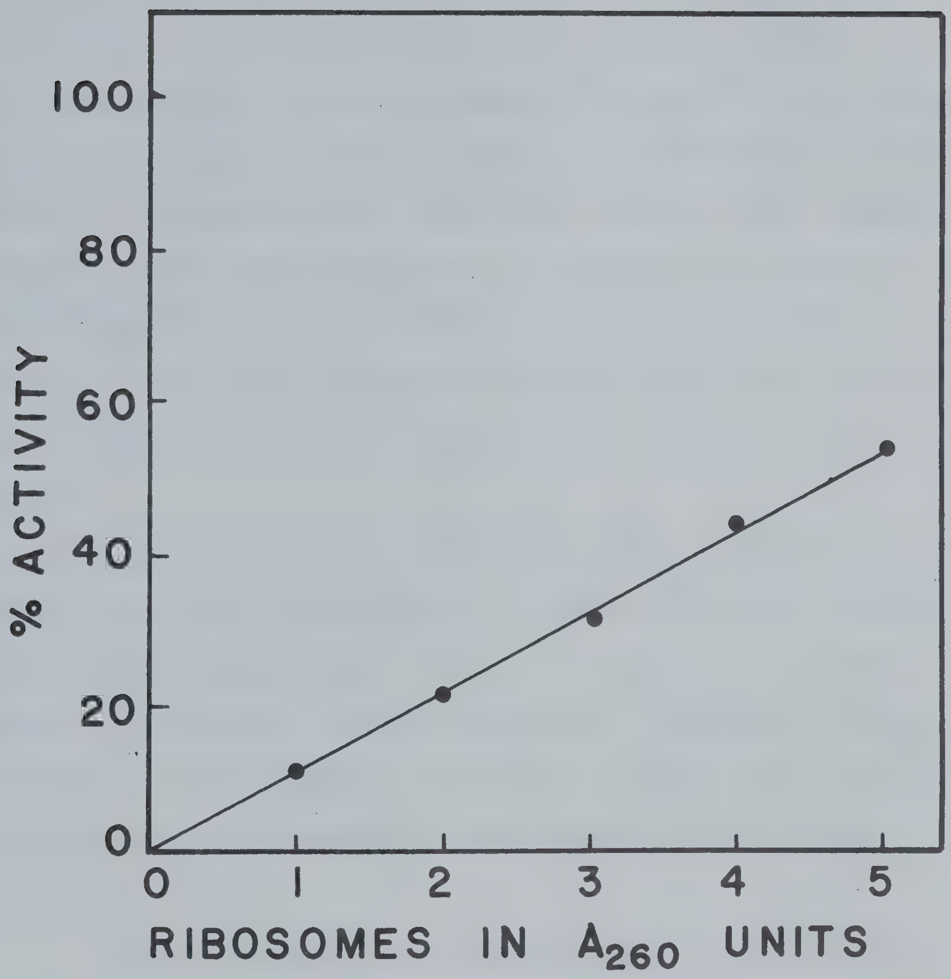


Fig. 6 Effect of ATA on poly-U:ribosome binding at various concentrations of ribosomes. The reaction mixture contained 20 μM ATA, 20 nmoles ^{3}H-poly-U phosphorus, and ribosomes as indicated on the abcissa. Reaction conditions and assay procedures are the same as those in Fig. 5. Percent poly-U bound (ordinate) indicates the calculation based on the radioactivity in the experimental set over the value in the control set (no ATA present) at a given concentration of ribosomes.

Buffer RM. As expected, these two components were excluded from the column due to their large molecular weight. ATA, on the other hand, was included in the column and thus was eluted with the included volume. Secondly, poly-U and ATA were mixed and subjected to column chromatography. The elution profile corresponded to that obtained when these components were chromatographed individually: that is, poly-U appeared in the void volume and ATA appeared in the included volume. However, when the mixture of ribosomes and ATA was chromatographed, there was only one ATA peak in the void volume and none in the included volume. This was taken to mean that the ATA had formed a complex with the ribosome.

An attempt was made to dissociate rabbit reticulocyte polysomes by ATA treatment into monomeric ribosomes and free mRNA. Although binding of ATA to the polysomes did occur as detected by a spectrophotometric measurement, there was essentially no dissociation of the polysomes even at ATA concentrations as high as 200 μM. This result indicated that a preformed mRNA:ribosome complex is protected from ATA inhibitory action.

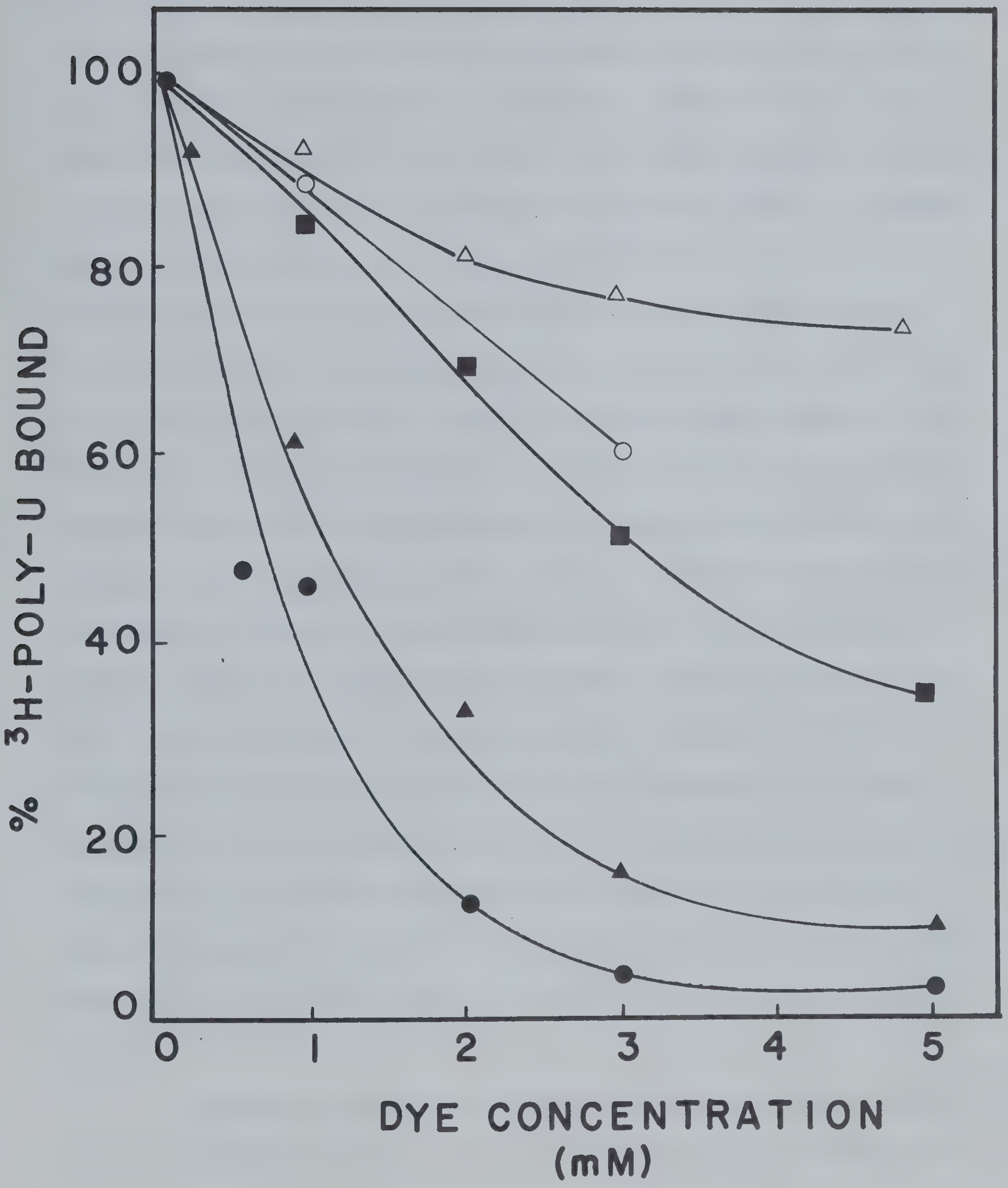
Although the above results indicated that the ribosome is the site of interaction with ATA, further studies with this system were complicated by the fact that the ribosome fraction used in these studies contained messenger RNA binding factors strongly associated with ribosomes (Igarashi and Dufresne 1974). It was therefore possible that ATA binds to the protein which mediates mRNA binding to the ribosome, or that it binds non-specifically to one or more of the structural components of the ribosome itself. The exact site of ATA action could thus only be determined by purifying the factors and

ribosomal proteins, and examining the interaction of ATA with each of them individually. However, at the time this study was undertaken, the identification of these factors and the individual ribosomal proteins had not yet been completed. Thus no further study on the site of ATA binding within the ribosomal structure or with factors was possible.

Comparative Study on the Inhibitory Action of Aurin Derivatives

An important aspect of ATA action in this system, yet to be explored, was the structural feature of ATA responsible for the observed inhibition. A series of aurin derivatives were chosen on the basis of salient differences in their side chain constitution, and tested for their capacity to inhibit the mRNA:ribosome binding reaction using the Millipore filtration method (see Fig. 1 for structures). It is obvious from Fig. 7 that these compounds inhibit mRNA:ribosome binding to some extent, but the I_{50} values are at least 50 times higher than that of ATA. However, it is noteworthy that the order of inhibitory power is: ATA (I_{50} = 20 μM),Aurin (I_{50} = 500 μM), Azure Blue B (I_{50}= 1500 μM). The foregoing results suggest that both carboxyl and hydroxyl groups are required for effective inhibition.

Fig. 7 Effect of various aurin derivatives on poly-U:ribosome binding. ^{3}H-poly-U (20 nmoles phosphorus equivalent) was mixed with ribosomes (5 A_{260} units) in the presence of various aurin derivatives, and the resulting complex was measured by the Millipore filtration method as described in the Methods. For each concentration of the individual dyes, a calculation based on radioactivity in the experimental set over the value in the control set (no dye present) was done. This value is shown in the figure as percent poly-U bound (ordinate) where 100% represents the control experiment. (●) Aurin, (▲) Azure Blue B, (■) Fuchsin Acidic, (△) Erichrome Cyanine, (○) Fuchsin Basic.


100
80
60
40
20
0
% ^{3}H-POLY-U BOUND
0
1
2
3
4
5
DYE CONCENTRATION
(mM)

(3-3) Discussion

The results reported in this chapter indicate that ATA inhibits both polyphenylalanine synthesis and poly-U binding to monosomes in the reticulocyte cell-free system. These results are in good agreement with the report of Grollman and Stewart (1968). However, the concentration required for 50% inhibition of polyphenylalanine synthesis was 5 μM, while that for 50% inhibition of poly-U binding to ribosomes was 20 μM. Thus ATA is four times as effective in the incorporation reaction than in the mRNA:ribosome binding reaction. This indicates that there may be steps in the overall protein synthesizing system sensitive to ATA in addition to the mRNA:ribosome binding reaction. This prediction can be supported by the recent reports of Siegelman (Siegelman and Apirion 1971, and Siegelman 1970) that various steps of protein synthesis can be inhibited by ATA. Further analysis of our system, as described in Chapter 4, revealed that among the individual steps of protein synthesis, aminoacyl-tRNA synthesis is strongly inhibited by ATA. Thus the very first reaction in protein synthesis, i.e. the formation of precursor is inhibited by ATA. This fact has not been reported in the literature to date and may be the cause for the higher sensitivity of the incorporation reaction toward ATA. Setting aside this new finding for discussion in the next chapter, the present discussion will concentrate on the dye effect on mRNA:ribosome binding itself.

In order to probe the mechanism of ATA action, three experimental approaches were taken. These included the effect of ATA on preformed polysomes, the relief of ATA inhibition by increasing the concentration of reaction components, and detection of stable complex

formation between ATA and a reaction component. One experiment, in which the order of adding reaction components was manipulated to see whether or not a preformed complex between ATA and a given reaction component changes the degree of ATA inhibition,showed that when ribosomes and poly-U were mixed prior to adding ATA, the inhibition was negligible. This fact indicates that ATA interferes only with a process leading to the stable complex formation between mRNA and ribosomes. In fact, when isolated polysomes from reticulocytes were incubated with ATA, there was virtually no dissociation of polysomes into monosomes. This resistance of polysomes towards ATA action agrees with the above observation that ATA is less effective on preformed poly-U:ribosome complexes. Recent studies (Rhoads et al 1973, Henshaw et al 1973, and Phong Cheng et al 1973) which support this conclusion, demonstrated that the addition of low levels of ATA does not affect the structure of the polysomes,at least for the time required to complete nascent polypeptide chains.

The second set of experiments was designed to determine the site of ATA action, and consisted of changing the concentration of individual reaction components in the presence of a fixed concentration of ATA. Early studies of Brown (1951) and Lindenbaum (1956) indicated that ATA would bind to proteins. However, no studies were reported examining the binding of ATA to RNA. The results reported here showed that varying the concentration of poly-U at a fixed concentration of ATA does not alter the effectiveness of the dye action, but that increasing the concentration of ribosomes showed a definite decrease in the ATA inhibition of poly-U binding to ribosomes. It is worth mentioning that the protein synthesizing reaction requires Mg^{++} as a cofactor.

Therefore, the possibility that the ATA inhibition is the result of chelating Mg^{++} should be considered carefully. The Mg^{++} concentration in the reaction mixture was 1 mM or higher depending on the reaction, whereas the effective concentration of ATA is between 5-20 μM. Thus, to significantly reduce the Mg^{++} concentration to produce the observed inhibition of the reaction, each ATA molecule would have to chelate at least 20 Mg^{++} ions. Such an occurance is chemically improbable and it was therefore concluded that the ribosomes are the site of ATA interaction in producing the inhibition of the mRNA binding.

The third set of experiments provided further support for the above conclusion. Gel exclusion chromatography of mixtures of ATA and poly-U or ATA and ribosomes showed that ATA binds to the ribosomes to form a stable complex which withstands the gel exclusion process. A similar stable complex between ATA and poly-U was not detected. Other laboratories have reported similar results (Grollman and Stewart 1970, and Heiberg et al 1971). It is thus clear that ATA binding to ribosomes causes an inhibition of the poly-U binding reaction. This observation gives rise to two questions: (1) what is the nature of ATA binding to the ribosomes? (2) how many ATA molecules will bind per ribosome? At this point,however, difficulties were encountered in our studies due to the complexity of the ribosome structure. Further delineation of the binding site must differentiate between ribosomal RNA, ribosomal protein, and the protein factors associated with the ribosomes. Separation and purification of these structural components of the ribosomes would be necessary to complete the assignment of the binding site of ATA which is directly responsible for inhibition of the mRNA binding reaction. An attempt was first made to prepare reticulocyte monosomes free of

poly-U binding factor using a method which was later applied to the rabbit liver monosomes successfully (Dufresne and Igarashi 1974). However, it was not effective in the reticulocyte system, since the method gave only about 50% removal of the poly-U binding factor as compared to 99% removal fron liver monosomes. The reason for this difference between liver and reticulocyte ribosomes is as yet unknown. Since the poly-U binding factor of the reticulocyte systemwas very strongly associated with the ribosome, it was not possible to carry out further studies on the nature of ATA binding to reticulocyte ribosomes.

The attempt to elucidate the nature of the ATA binding site was limited to the extent just described in the preceeding section. However, one more important aspect of the inhibitor study must be considered, i.e. the structural element of ATA responsible for the observed inhibition. In this regard, the effect of various aurin derivatives on the mRNA:ribosome binding reaction was examined. The results showed that all of the compounds tested are capable of inhibiting the reaction to some extent. They are,however, much less effective than ATA—a concentration of at least 25 times that of ATA is required to produce a comparable level of inhibition. The analysis of these data allows us to probe the mechanism of ATA action one step further. The aurin derivatives with I_{50} values closest to that of ATA are Aurin and Azure Blue B. A common feature of these two compounds is that they are negatively charged at physiological pH. It is known that ATA forms 'lakes' by complexing with metal ions (Lisenko _et_ _al_ 1971). Therefore the possibility of chelation of the Mg^{++} ion once again comes to light. However, as stated above, one ATA molecule must chelate at least 25 Mg^{++} ions under

the experimental conditions used. This is physically impossible and therefore, the mechanism of ATA action cannot be chelation.

This conclusion led us to a close examination of the structure of ATA, Aurin and Azure Blue B. It is evident that the important functional groups common to these compounds are hydroxyl and/or carboxyl groups. These groups are not found on the other triphenylmethanes tested—Fuchsin Basic, Fuchsin Acid and Erichrome Cyanine— which are less effective inhibitors of the reaction, with I_{50} values of greater than 5 mM. From this observation, we can conclude that the presence of hydroxyl and/or carboxyl groups as functional groups has a role in the inhibitory action. The chemical reason for this apparent requirement is as yet unclear.

These early studies on the ATA inhibition led us to a series of studies on the effect of this compound on various enzymic reactions. The following chapter describes one of these studies involving the effect of ATA on the phenylalanyl-tRNA synthetase reaction.

CHAPTER 4

THE EFFECT OF ATA AND AURIN DERIVATIVES ON PHENYLALANYL-tRNA SYNTHETASE REACTION

(4-1) Introduction

The studies described in Chapter 3 led us to speculate that there must be some ATA sensitive reaction other than the mRNA:ribosome interaction in the overall protein synthesizing process. This prediction is based on the observation that ATA is more effective in inhibiting the overall incorporation of phenylalanine than in the more specific mRNA:ribosome binding reaction. Thus the search for at least one more ATA sensitive reaction was conducted. In the course of the incorporation reaction, we always set aside an aliquot of the reaction mixture for detection of aminoacyl-tRNA synthesis to ensure the formation of sufficient precursor molecules for the reaction. The results indicated that, in the presence of ATA, aminoacyl-tRNA synthesis is markedly reduced. The inhibitory effect of ATA on the aminoacyl-tRNA synthetase reaction has not previously been reported. Therefore, the study of the mechanism of ATA inhibition of the phenylalanyl-tRNA synthesis. was undertaken (Igarashi and Zmean 1974).

At the time this study commenced, the nature of aminoacylation reactions was well established for both bacterial and mammalian systems. It is a two-step reaction involving the formation of aminoacyl-AMP and the subsequent transfer of the aminoacyl moiety to a specific receptor tRNA. This two-step reaction is catalyzed by a single enzyme specific to each amino acid (Novelli 1967). However, the structure of the enzyme from mammalian origins was not fully elucidated at that time.

The overall molecular weights of aminoacyl-tRNA synthetase vary extensively (Lanks et al 1971, Fasiolo et al 1970, and Weinstein et al 1971). Moreover, the subunit structure of the enzymes varies considerably to give rise to either $\alpha 2$ or $\alpha 2\beta 2$ forms (Le Meur et al 1972, Penneys et al 1974, Fasiolo et al 1970, and Tscherene et al 1973). The present investigation on the action of ATA on the phenylalanyl-tRNA synthetase reaction included: the determination of the inhibitory concentration of ATA, the determination of the structural features responsible for the observed inhibition, and the stoichiometry of the ATA:enzyme interaction. Although the lack of precise information regarding the molecular weight and subunit structure of phenylalanyl-tRNA synthetase introduced some ambiguity into these analyses, the studies nonetheless led us to conclude that ATA is a non-specific inhibitor which interacts not only with ribosomes but also with aminoacyl-tRNA synthetase.

(4-2) Results

Effect of ATA on the Aminoacyl-tRNA Synthetase Reaction

In order to study the effect of ATA on the synthetase reaction, it was necessary to establish the optimal conditions for this reaction. In particular, the effect of salt concentration required special attention because the crude reticulocyte synthetase fraction was precipitated by ammoniun sulfate at 66% saturation and suspended in an equal volume of Buffer A. Thus this fraction contained a significant amount of monovalent cations necessitating an adjustment in the standard assay, depending on the volume of enzyme solution added. Fig. 8 shows that the optimal concentration of KCl is 30 mM and of $MgCl_2$ is 12 mM when the ATP concentration is 1 mM. The addition of β-mercaptoethanol at 1 mM is also essential to the reaction. Based on this data, the synthetase

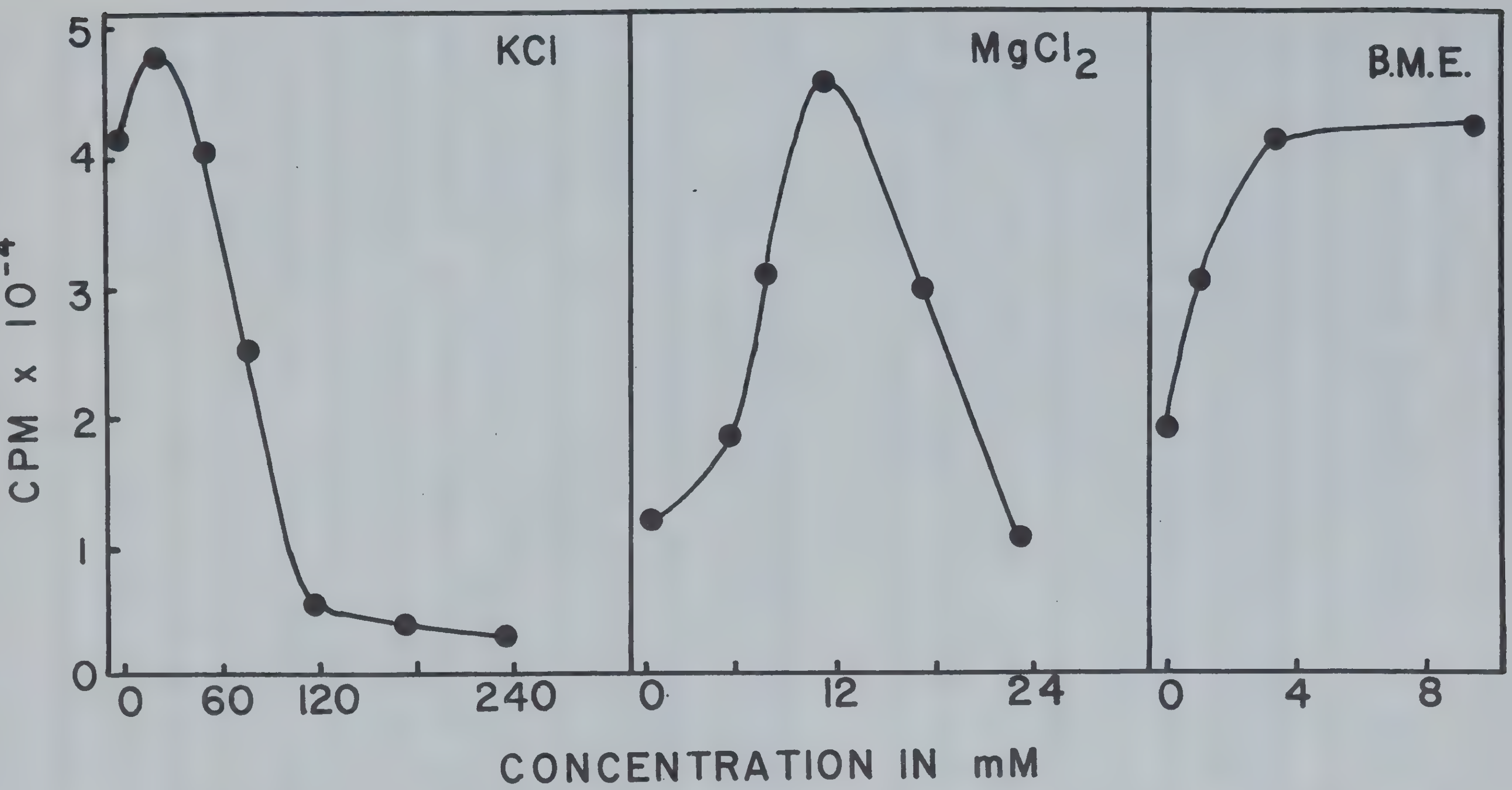


Fig. 8 Effect of salt concentrations on ^{3}H-phenylalanyl-tRNA synthesis. Assays were performed using 0.1 ml reaction mixture as described in Methods 2-11. The concentrations of KCl, $MgCl_2$, and βmercapto-ethanol were individually altered as indicated on the abscissa, while the concentrations of tRNA(0.1 mg), ATP (0.1 μmoles), and AS66 enzyme were held constant. ^{3}H-phenylalanyl-tRNA synthesized at 20 min of reaction at 37°C was plotted on the ordinate.

reactions in the following experiments were carried out as specified in the Methods (2-11).

The effect of various concentrations of ATA on the phenylalanyl-tRNA synthetase reaction was then studied using the rabbit reticulocyte synthetase ,fraction AS66. The complete reaction mixture (see Methods) included various concentrations of ATA and was incubated at 37°C for 20 min. The samples were processed with the cold TCA wash as described in the Methods. The level of ^{3}H-Phe-tRNA synthesized after 20 min reaction in the presence of ATA was compared with that in the control set, which contained no ATA, and expressed as percent activity. Fig. 9 demonstrates that ATA inhibits the Phe-tRNA synthetase reaction, reducing it by half at a concentration of 50 μM (I_{50} = 50 μM).

Effect of Aurin Derivatives on the Phe-tRNA Synthetase Reaction

In order to investigate the nature of the ATA inhibition of the phenylalanyl-tRNA synthetase reaction, an inhibitor study was conducted using aurin derivatives. The I_{50} level for each of the triphenylmethanes tested was established using the method previously described for ATA. The structures of these compounds are given in Fig. 1. Table 1 shows the I_{50} values of the aurin derivatives tested for the phenylalanyl-tRNA synthetase reaction. It is clear that all of the compounds tested are potent inhibitors of the aminoacyl-tRNA synthetase reaction unlike that observed for the mRNA:ribosome binding reaction described in Chapter 3. It must be pointed out that the charge properties of the side chains are not critical in this system since both Aurin (negatively charged) and Fuchsin Basic (positively charged) exhibit inhibitory action at approximately the same concentration—250 μM. It is also noticeable that compounds possessing carboxyl groups are

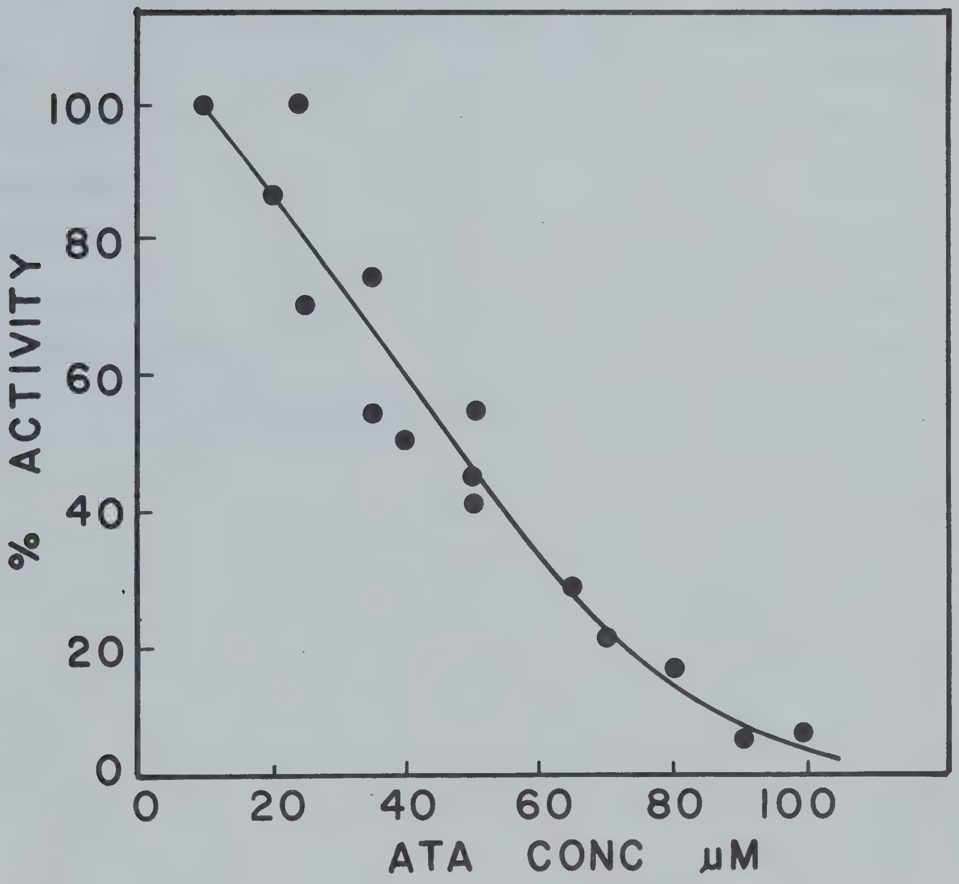


Fig. 9 Inhibition of phenylalanyl-tRNA synthetase by ATA. Assays were performed as given in the Methods. The incorporation of ^{3}H-phenylalanine into ^{3}H-phenylalanyl-tRNA at 20 min incubation was taken as 100% activity. The activity in the presence of varying amounts of ATA is indicated as the percent of activity relative to the control value. (100% = 4.2 pmoles Phe). The protein concentration was 100 µg per 0.1 ml reaction mixture.

Table 1

Inhibition of Phe-tRNA Synthesis by Aurin Derivatives

Aurin Derivative	I_{50} Value
Aurin	250 μM
Fuchsin Acid	250 μM
Fuchsin Basic	250 μM
Patent Blue	150 μM
Azure Blue B	50 μM
Aurintricarboxylic acid	50 μM

slightly stronger inhibitors than those possessing sulfonyl groups. A reduction in the number of conjugated resonance groups from three (ATA) to two (Azure Blue B) does not alter the inhibitory effect at all. These observations suggested to us that neither the skeletal structure of triphenylmethanes nor the side chains are important for their inhibitory action. This information then suggested to us that the aurin derivatives may inhibit this reaction through a different mechanism from that observed in protein synthesis. It is possible that the phenyl group of the aurin derivatives itself may compete with phenylalanine towards its binding site. However, this possibility is remote since all of the aminoacyl-tRNA synthetase reactions tested were inhibited by ATA. A more detailed analysis of the mode of action will be dealt with in a later section.

Site of ATA Action on the Phe-tRNA Synthetase Reaction

In order to investigate the possibility that ATA exerts its inhibitory action by interacting with one of the reaction components, a preincubation study was undertaken. When ATA at a concentration of 20 μM was preincubated 5 min at 4°C with yeast tRNA, the subsequent assay for Phe-tRNA synthesis at 37°C showed 88% activity as illustrated in Table 2. When ATA was preincubated with both the enzyme and tRNA, 90% activity was shown. When ATA was preincubated with the enzyme alone, 76% activity was detected. These results show that preincubation of ATA with the enzyme fraction produces a slightly higher inhibition. However, they do not clearly distinguish whether the site of inhibition is tRNA or the enzyme.

In order to provide more definitive data, a concentration

Table 2

Change in ATA Inhibition by Differential Preincubation of Reaction Components for Phe-tRNA Synthesis

Preincubation Mixture	% Activity
ATA + enzyme	76
ATA + tRNA	88
ATA + tRNA + enzyme	90

effect was examined, where the concentration of one reaction component was altered while the concentration of the other components and ATA (50 μM) remained constant. If changing the concentration of a substance alters the level of inhibition produced by a given concentration of the inhibitor, then it is an indication that that substance is the site of the inhibitory action. The four reaction components of the synthetase reaction were examined—tRNA, ATP, Mg^{++}, and synthetase. Fig. 10 shows the effect of varying the tRNA concentration at a constant level of ATA. The curve shows essentially the same percent activity whether 30 or 300 μg of tRNA are added to 0.1 ml of reaction mixture. From these results, we conclude that the concentration of tRNA does not have a pronounced effect on the inhibition caused by ATA. Similarly, changes in the ATP and Mg^{++} concentrations did not alter the level of inhibition achieved by ATA at a concentration of 50 μM. However, in the case of the experiment in which synthetase concentration was varied, the results were much different from those described above. Fig. 11 demonstrates that, by raising the concentration of synthetase above 100 μg per reaction mixture in the presence of 50 μM ATA, inhibition levels of less than 50% are observed. In fact, raising the concentration of the enzyme to 150 μg or higher per reaction mixture effectively restores the level of ^{3}H-Phe-tRNA formation to 100% in the presence of 50 μM ATA. Conversely, lowering the concentration of synthetase to levels below 100 μg produces levels of inhibition greater than 50%. Of the reaction components tested, only synthetase showed such a concentration effect. These results clearly show that increasing the enzyme concentration serves to overcome the ATA inhibition. Therefore, we drew a tentative conclusion that ATA interacts in some way with

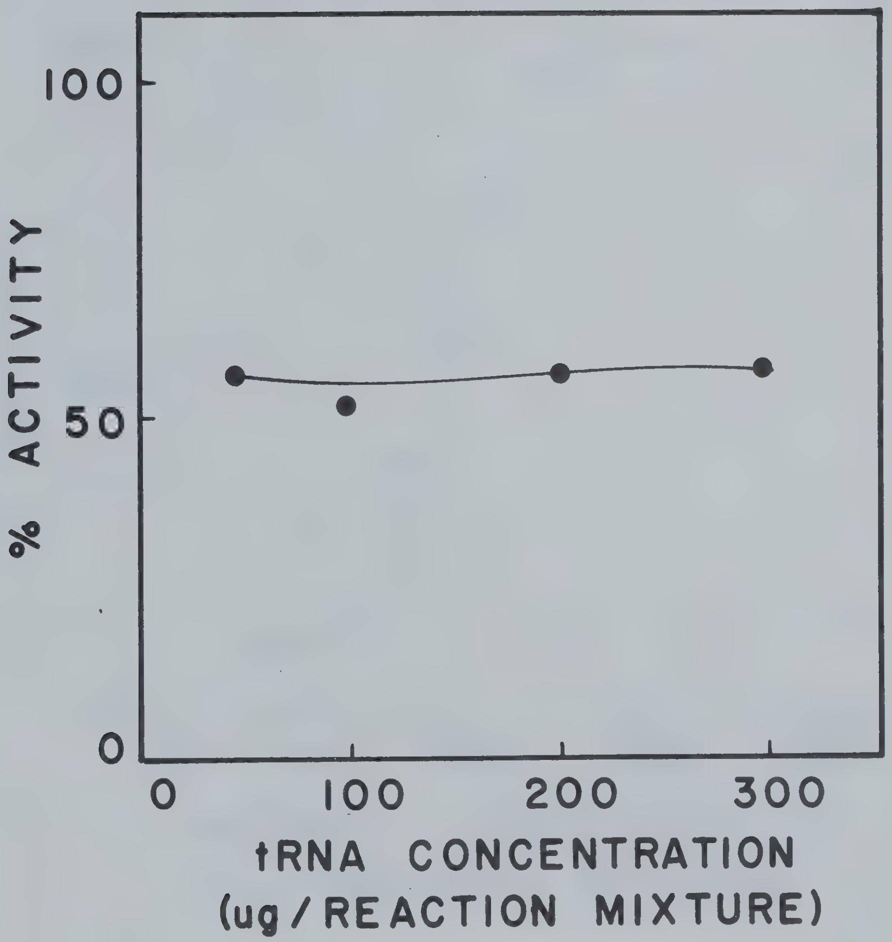


Fig. 10 Effect of tRNA concentration on inhibition of Phe-tRNA formation by ATA. Assays were performed as described in the Methods, except that tRNA was added to start the reaction. Phe-tRNA synthesis at 20 min of reaction in the absence of ATA was taken as 100% activity. The level of activity for each tRNA concentration in the presence of 50 μM ATA was measured, and relative activity to the control value (for that tRNA concentration) was expressed as percent activity.

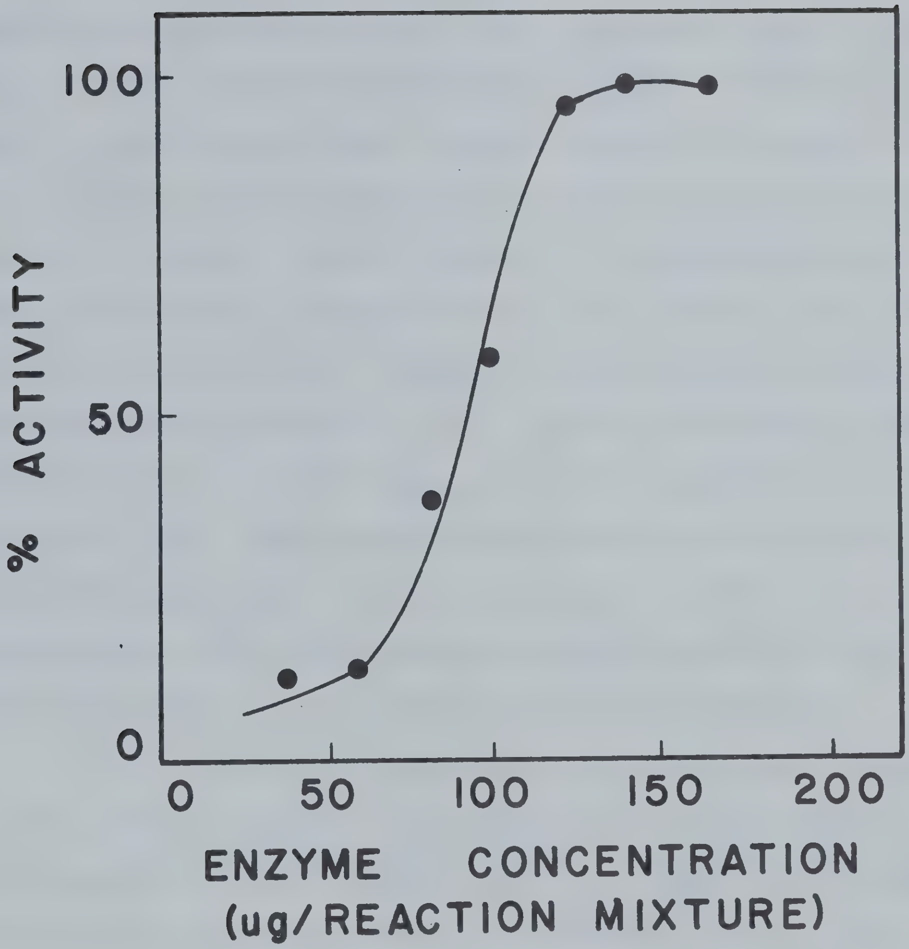


Fig. 11 Effect of the concentration of Phe-tRNA synthetase on the inhibition of Phe-tRNA formation by ATA. Assays were performed as described in the Methods, except that tRNA was added last to start the reaction. The ^{3}H-Phe-tRNA synthesis at 20 min of reaction in the absence of ATA for each enzyme concentration was taken as 100% activity. The level of activity for each enzyme concentration in the presence of 50 μM ATA was measured and the percent activity calculated as in Fig. 10.

phenylalanyl-tRNA synthetase.

Binding of ATA to Phe-tRNA Synthetase

If the observed inhibitory action of ATA on the Phe-tRNA synthetase reaction is truly caused by the interaction of ATA with the enzyme, a stable complex formation between them may be detected as was done in the mRNA:ribosome binding reaction. Initially, an attempt was made to separate the enzyme:ATA complex by a molecular seiving method as was done in Chapter 3 for the detection of a ribosome:ATA complex. However, a problem was encountered due to the fact that the AS66 enzyme contained a large proportion of hemoglobin which possesses an absorption spectrum overlapping with that of ATA. In addition, the molecular weight of hemoglobin is in a similar range to that of the synthetase—as will be described later. These problems prevented adequate measurement of the ATA:synthetase complex. Therefore, purification of Phe-tRNA synthetase was required. After achieving the removal of hemoglobin as described in the Methods, the following experiments were performed.

First of all, the I_{50} value had to be re-established for the partially purified enzyme at a level of 100 μg per reaction mixture and in the presence of various concentrations of ATA. Assays were performed as given in the Methods. The level of ^{3}H-Phe-tRNA synthesis in the presence of various concentrations of ATA was compared. From Fig. 12, the I_{50} value for the partially purified enzyme was estimated to be 13.5 μM. This value represents a shift in I_{50} from that for the crude Phe-tRNA synthetase (AS66) fraction. It should be mentioned that

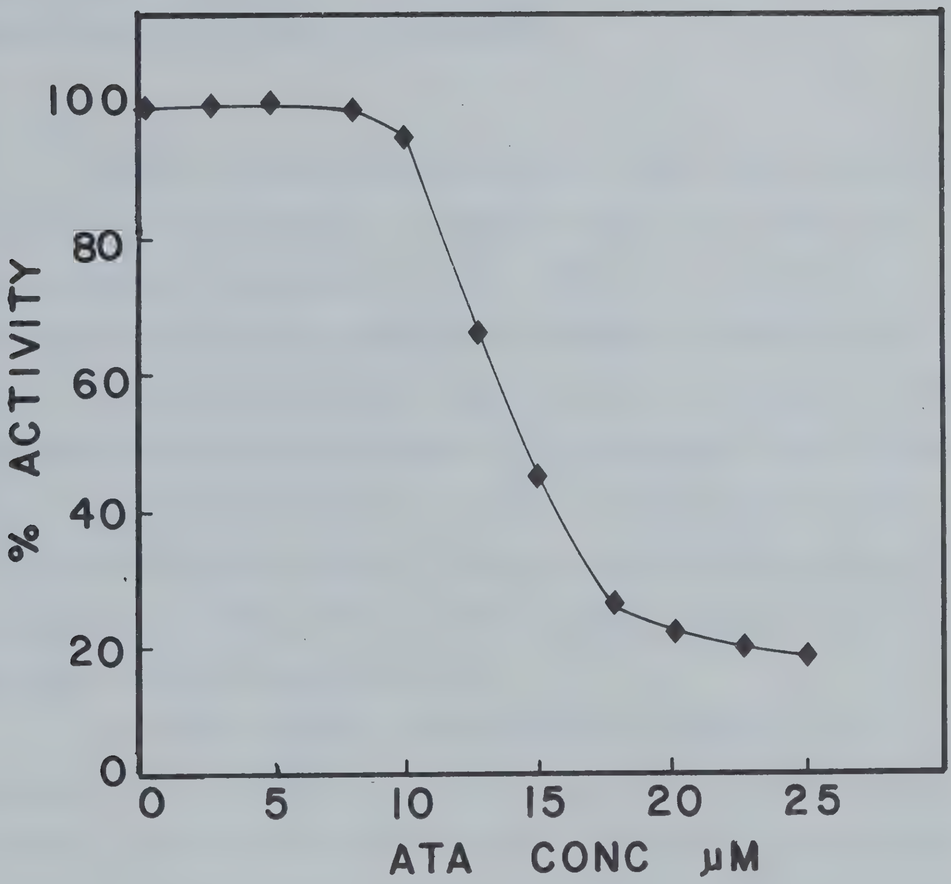


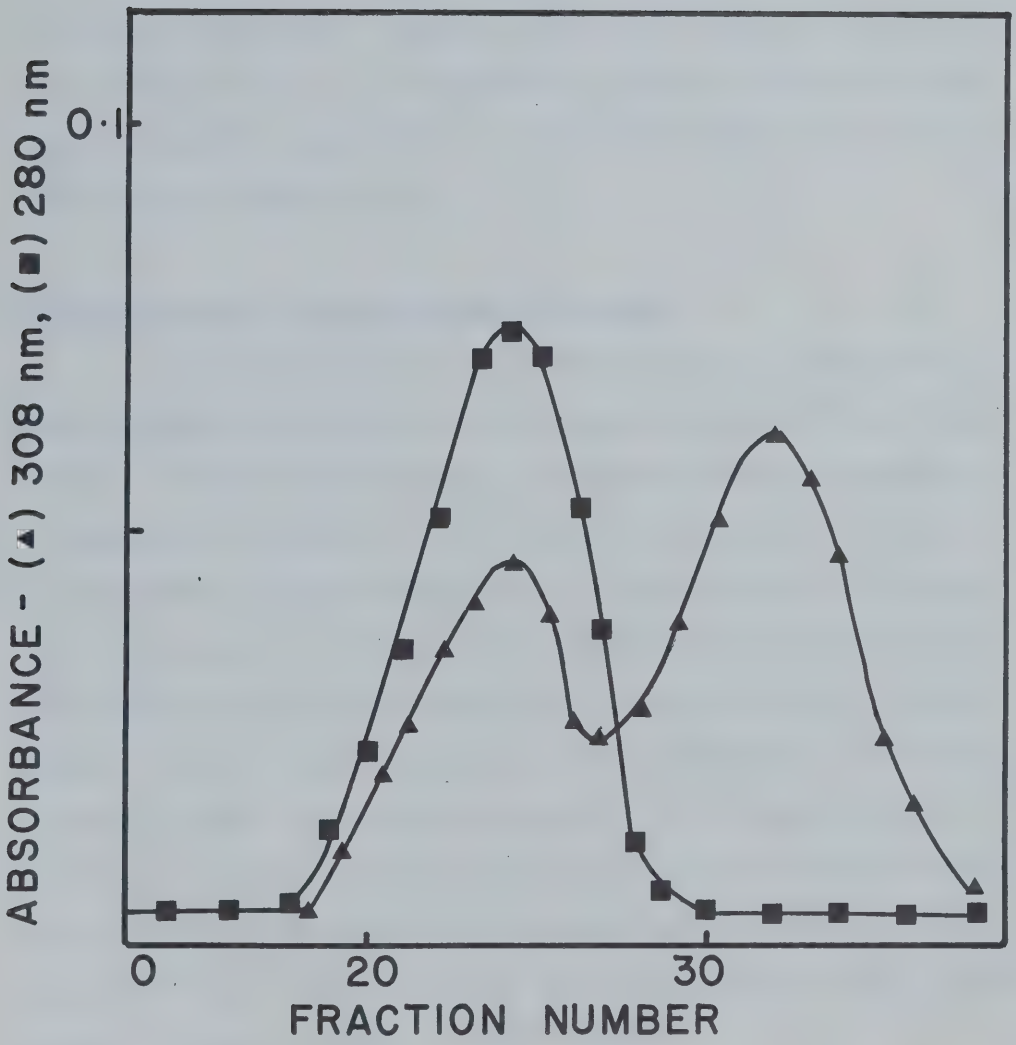
Fig. 12 Inhibition of partially purified Phe-tRNA synthetase by ATA. Assays were performed as given in the Methods. The incorporation of ^{3}H-Phe into ^{3}H-Phe-tRNA at 20 min of reaction in the absence of dye was taken as 100% activity (30 pmoles Phe). Varying amounts of ATA were added, the activity measured, and the results were expressed as a percent of the control activity. The protein concentration was 20 µg per 0.1 ml of reaction mixture.

the ratio of these two I_{50} values (approximately 4) is very close to the ratio of protein concentrations used in these two sets of experiments: 100 µg/20 µg = 5. It thus appears that the reduction in the I_{50} value of the partially purified enzyme is due to the reduction in protein concentration. This is what one would expect if ATA has affinity toward many proteins to about the same extent.

In order to detect the formation of a stable complex between ATA and the Phe-tRNA synthetase, a mixture of ATA and the enzyme was chromatographed on a Sepharose 6B column. The elution profile of the column was determined by an assay of each fraction for synthetase activity. The peak position of the enzymatic activity coincided with that for a protein fraction as monitored by 280 nm absorption. This indicated that the enzyme was reasonably pure in terms of charge properties and of molecular weight. The position of ATA was then determined by monitoring absorption at 308 nm, which provides a minimum overlap with the absorption spectrum of protein and RNA.

To determine whether or not ATA binds to synthetase, a solution of 500 µg enzyme in Buffer RM was made to 40 µM in ATA. Fifty microliters of this mixture was loaded onto a Sepharose 6B column of bed volume 5 ml which was pre-equilibrated with Buffer RM. Five drop fractions of volume 0.175 ml were collected and diluted to 0.5 ml with water. Each fraction was examined for absorption at 280 nm and 308 nm using a spectrophotometer. These results, illustrated in Fig. 13, show that there is significant absorbance at 308 nm at the position which

Fig. 13 Sepharose 6B analysis of partially purified Phe-tRNA synthetase: ATA mixture. A solution containing 500 μg enzyme and 40 μM in ATA in 50 μl was layered onto a Sepharose 6B column of bed volume 5 ml (5 mm x 130 mm). Materials were eluted from the column using Buffer RM. Five drop fractions (0.175 ml) were collected and diluted with water to a volume of 0.5 ml. Thereafter, absorption at 280 nm for the enzyme and at 308 nm for ATA was determined for each fraction. Absorbance at 280 nm (■) Absorbance at 308 nm (▲).


ABSORBANCE - (▲) 308 nm, (■) 280 nm
0·1
0
20
30
FRACTION NUMBER

corresponds to the enzyme peak. This indicates that ATA binds to the enzyme to form a stable complex detectable by column chromatography.

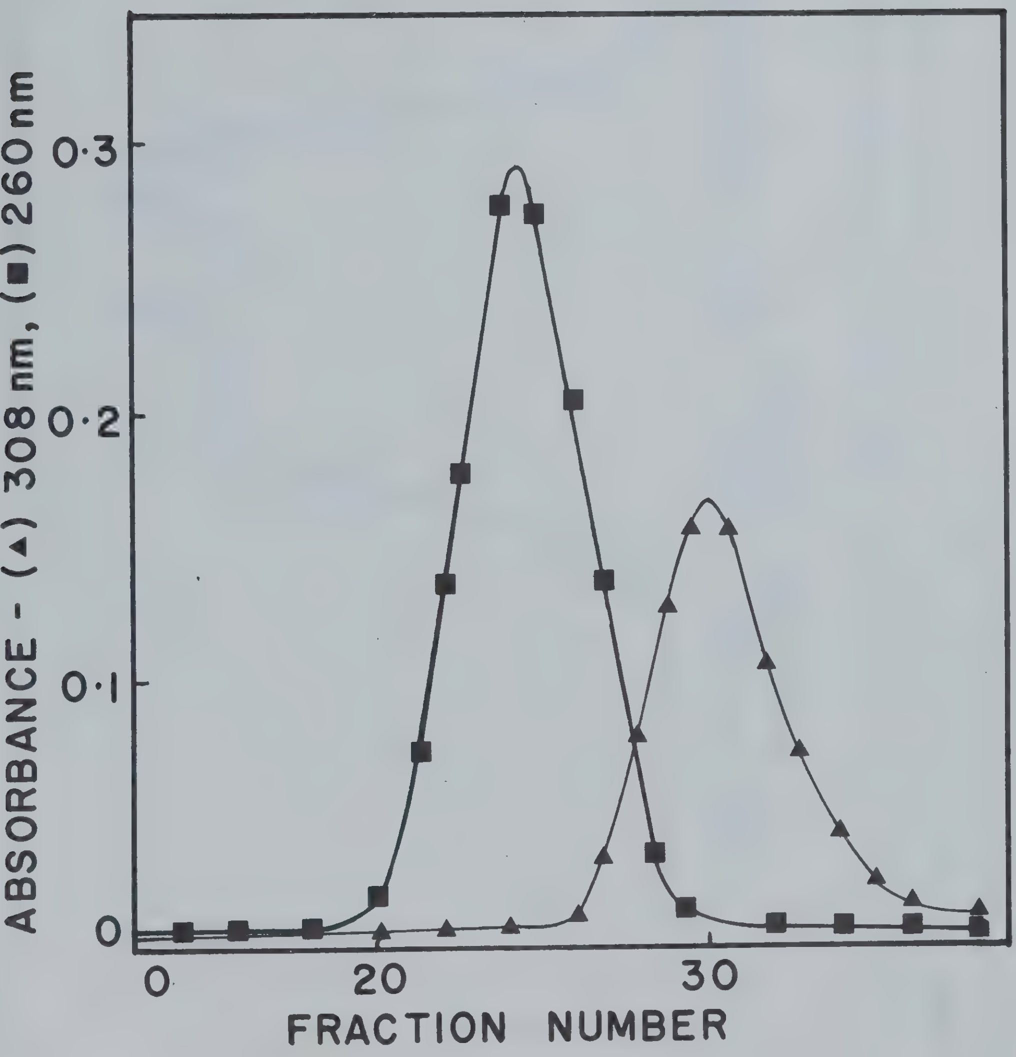
A similar experiment was then performed using a mixture of yeast tRNA and ATA. For this study, 50 µg of tRNA was made to 40 µM ATA in Buffer RM. The procedure used was as described above except that the diluted samples were examined at 260 nm for tRNA and at 308 nm for ATA. As shown in Fig. 14, no absorbance at 308 nm occurs in the tRNA region. From this observation, we conclude that tRNA does not bind to form a stable complex with ATA.

Semiquantitation of ATA:Synthetase Interaction

As the first step in determining how many ATA molecules can bind per enzyme molecule, it was necessary to determine the molecular weight of the Phe-tRNA synthetase. This enzyme apparently migrated as a single peak on Sepharose 6B as detected by assay of the fractions for Phe-tRNA synthetase activity. Fig. 15 shows the relative elution pattern of marker proteins of known molecular weight and the enzyme. The molecular weights of the marker proteins are as follows: Glucose oxidase, 186,000; Chymotrypsinogen A, 25,000; Hemoglobin, 68,000; and Lysozyme, 14,000. Fig. 16 shows the replot of the data from Fig. 15. There is a linear relationship between the log of the molecular weight and the elution positions of the individual proteins from the column. From this data, the position of the Phe-tRNA synthetase activity corresponds to a molecular weight of 70,000 daltons.

The next step was to calculate the number of ATA molecules bound per molecule of Phe-tRNA synthetase. Tube #24 from Fig. 13, containing the enzyme:ATA, was used for this purpose. The amount of enzyme

Fig.14 Sepharose 6B analysis of tRNA-ATA mixture. Fifty microliters of a tRNA: ATA mixture (50 µg yeast tRNA, 40 µM ATA) was layered onto a Sepharose 6B column of bed volume 5 ml (5 mm x 130 mm). Materials were eluted from the column using Buffer RM and five-drop fractions were collected (volume 0.175 ml). Samples were diluted to 0.5 ml and examined for absorption at 260 nm for tRNA and at 308 nm for ATA. Absorbance at 260 nm (■) Absorbance at 308 nm (▲).


ABSORBANCE - (▲) 308 nm, (■) 260 nm
0·3
0·2
0·1
0
0
20
30
FRACTION NUMBER

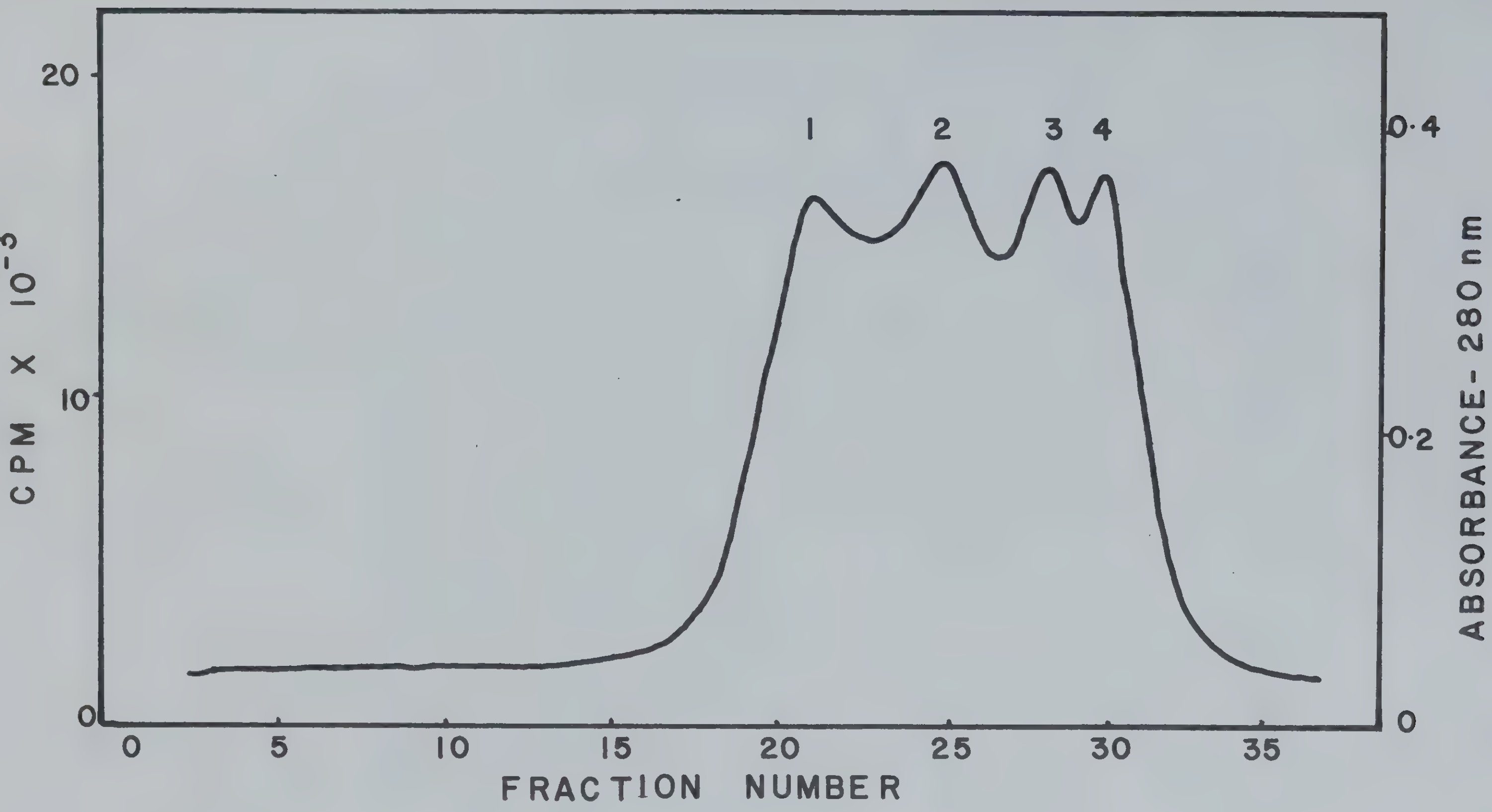
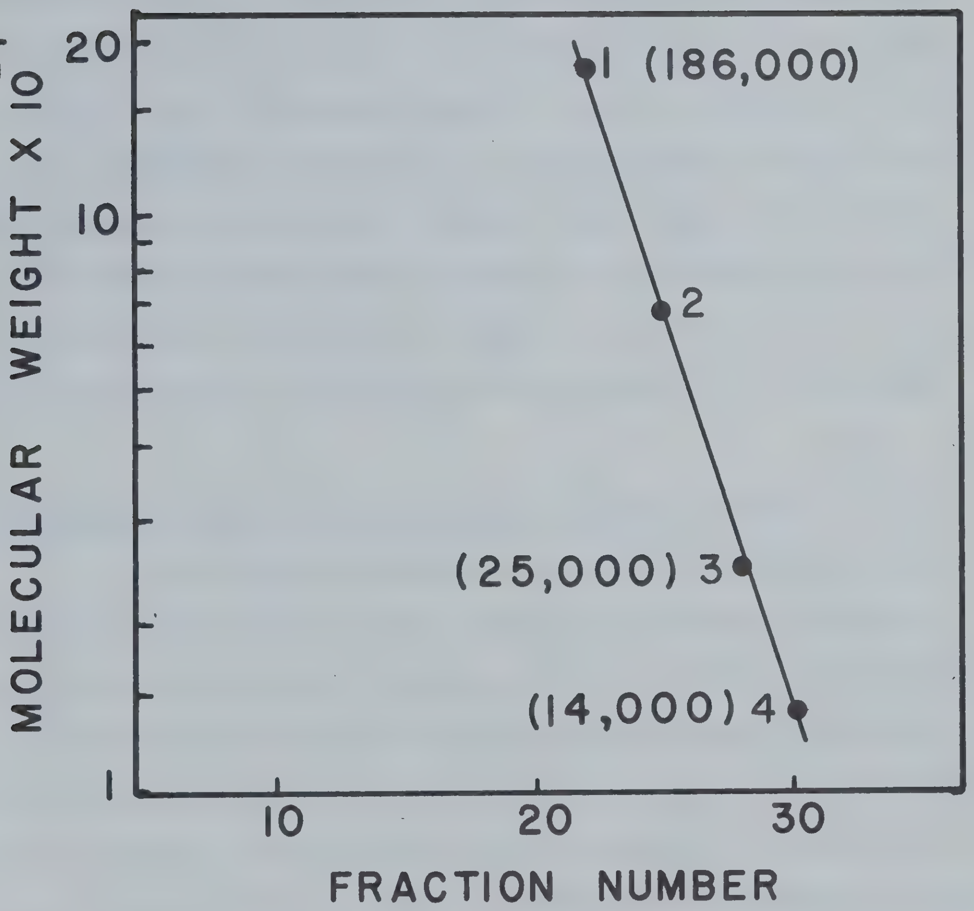


Fig. 15 Elution profile of the marker proteins and Phe-tRNA synthetase from Sepharose 6B. The position and molecular weight of each protein is as follows: 1. Glucose oxidase,186,000; 2. Phe-tRNA synthetase; 3. Chymotrypsinogen A,25,000; and 4. Lysozyme,14,000.

Fig. 16 Molecular weight estimation of partially purified Phe-tRNA synthetase on a Sepharose 6 B column. The relative positions of marker proteins and the enzyme, as determined in Fig. 15, are plotted against the molecular weight in the logarithmic scale. The molecular weight of each marker protein used is inserted in the figure: 1. Glucose oxidase, 186,000; 2. Phe-tRNA synthetase; 3. Chymotrypsinogen A, 25,000;and 4. Lysozyme,14,000. The peak positions were determined by 280 nm absorbance except in the case of the synthetase where the peak position was determined by assay of the fractions for synthetase activity. This data gave rise to a molecular weight of 70,000 daltons for Phe-tRNA synthetase.


MOLECULAR WEIGHT X 10^{-4}
20
10
1
10
20
30
FRACTION NUMBER
1 (186,000)
2
(25,000) 3
(14,000) 4

present in the tube was calculated from its absorbance data using an extinction coefficient of 1 A_{280}/mg/ml/10 mm light path together with the estimated molecular weight of the enzyme as 70,000. The amount of ATA present in the tube was determined from its absorbance at 308 nm using its extinction coefficient of 15.2 A_{308}/mg/ml/10 mm light path. The result of these calculations indicated that for each enzyme molecule, there are 6 ATA molecules present. Since the extinction coefficient of Phe-tRNA synthetase had not been established, the above value may be slightly different from the true value. However, extinction coefficients of various natural proteins deviate between 0.4 to 1.2 (Wetlaufer 1962). Therefore, the number of ATA molecules bound per molecule of the enzyme would range from 2.5 to 7 molecules.

The Mode of Inhibition in the Phe-tRNA Synthetase Reaction

It is valuable to determine the effect of ATA:enzyme complex formation of the binding of other ligands involved in the reaction. Based on the finding that Phe-tRNA synthetase can be retained on Millipore filters under appropriate conditions (see Methods), the effect of ATA on tRNA binding was studied using radioactively labelled yeast tRNA. A mixture of the partially purified enzyme and ^{3}H-tRNA was tested for binding to Millipore filters in the presence and absence of ATA (30 μM). The amount of ^{3}H-tRNA bound to the enzyme in the presence of ATA was reduced to 30% relative to that in the absence of ATA. The results shown in Table 3 indicate that ATA does interfere with the binding of tRNA to Phe-tRNA synthetase. However, the result does not distinguish the possible mechanism of inhibition due to either direct insertion of the ATA molecule into the tRNA binding site or an indirect

Table 3

Binding of ^{3}H-tRNA to Phenylalanyl-tRNA Synthetase

Reaction mixture	CPM Retained
^{3}H-tRNA alone	394
^{3}H-tRNA + enzyme	12,854
^{3}H-tRNA + enzyme + ATA (30 μM)	3,978

effect. An attempt was made to study the effect of ATA on ATP and phenylalanine binding to the enzyme under the same conditions as described above. However, there was no detectable complex formation between these ligands and the enzyme on Millipore filters. Their dissociation constants must be much lower than that of tRNA.

Because of the limited information available from the above experiment, we analyzed some already available data somewhat differently to probe the mode of ATA inhibition. Namely, the data given in Fig. 10 ,concerning the effect of substrate concentration on ATA inhibition, was submitted to a Lineweaver-Burke plot (plot of the reciprocal of the initial velocity vs. the reciprocal of the concentration of a substrate when the concentrations of the other substrates are fixed). Fig. 17 clearly indicates that ATA is a non-competetive inhibitor for tRNA. This analysis led us to conclude that ATA binds to the synthetase at a site other than the tRNA binding site.

(4-3) Discussion

The results reported in this chapter indicate that ATA inhibits Phe-tRNA synthesis at a concentration of 50 μM. This reaction is not more sensitive than the mRNA:ribosome binding reaction (I_{50} = 20 μM). Nevertheless, inhibition of Phe-tRNA synthesis by ATA has not been previously reported, and the study of ATA action in this system could provide some information about the nature of its action.

In order to probe the mechanism of ATA inhibition in the Phe-tRNA synthetase reaction, an experimental approach similar to that used in Chapter 3 for the mRNA:ribosome binding reaction was employed. First of all, the structural requirement for the described inhibition by ATA

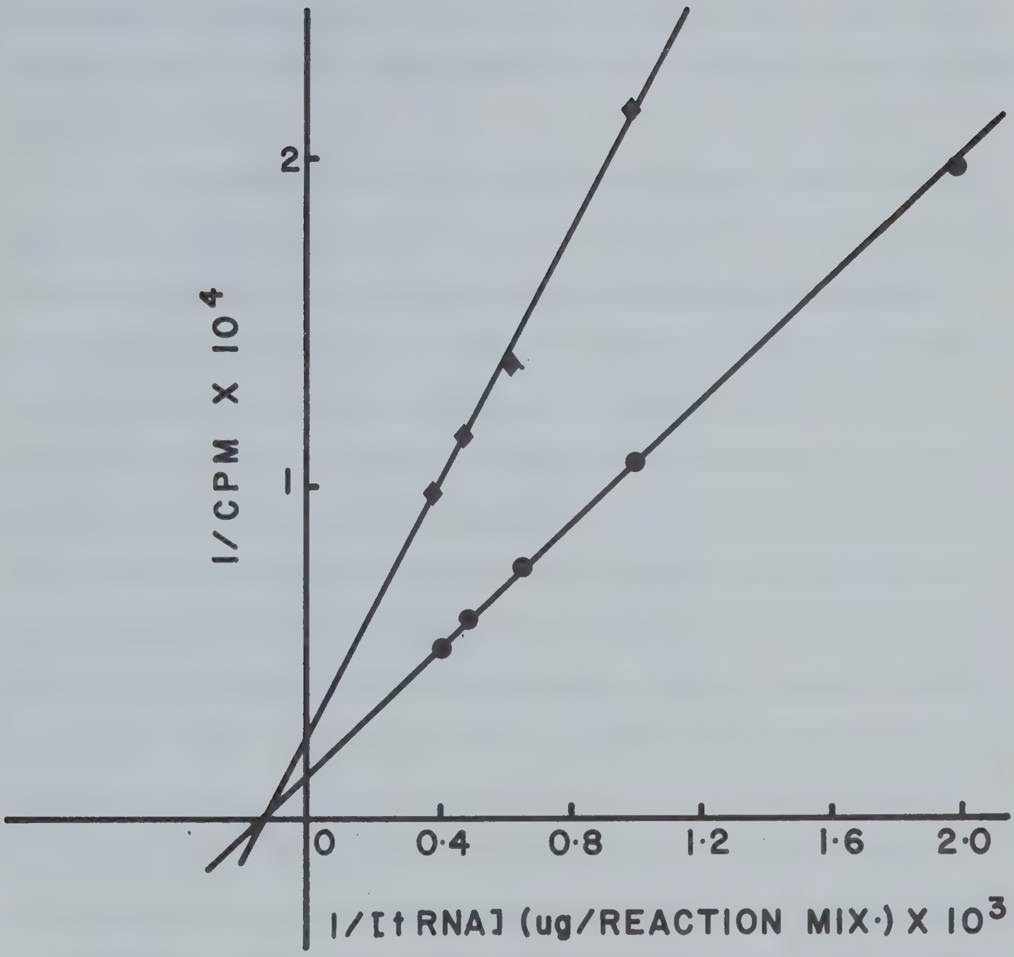


Fig. 17 Lineweaver-Burke plot of the phenylalanyl-tRNA synthetase reaction from Fig. 10: the reciprocal of the initial velocity against the reciprocal of tRNA concentrations in the presence and absence of a fixed level of ATA. (●) no ATA, (◆) 30 μM ATA.

was sought by comparing the relative inhibition by various aurin derivatives. Then the site of ATA inhibition in the synthetase reaction was studied in detail.

A comparative study with aurin derivatives indicated that all of the compounds tested were potent inhibitors of the reaction. This finding is quite different from what was observed in the mRNA: ribosome binding reaction as reported in Chapter 3 and suggests that the mode of ATA action may be different in the two reaction systems. The fact that Aurin (negatively charged) and Fuchsin Basic (positively charged) inhibit the Phe-tRNA synthetase reaction at the same concentration of 250 μM, clearly indicates that the charge of the molecule is not important for this inhibitory action. However, the type of substituting groups present on the triphenylmethane skeleton appears to affect the level of their inhibition. Those compounds possessing hydroxyl groups are effective inhibitors. In addition, the carboxyl group enhances the inhibitory action. For example, Aurin, which has three hydroxyl and no carboxyl groups has an I_{50} = 150 μM, while, ATA, which has three hydroxyl and three carboxyl groups has an I_{50} = 50 μM. Thus the presence of both hydroxyl and carboxyl groups gives rise to the strongest inhibitory action on this enzyme system.

The study of the mechanism of ATA action in the Phe-tRNA synthesis reaction included:the addition of reaction components in different order, relief of ATA inhibition by increasing the concentration of reaction components, and detection of a stable complex formation between ATA and individual reaction components. By changing the order of adding the reaction components, it was hoped to probe the site of ATA interaction with a specific reaction component. The results showed

that when ATA and tRNA were mixed prior to addition of the enzyme, the inhibition was lower than when ATA and the enzyme were mixed prior to the addition of tRNA. This indicates that the inhibitory action of ATA is more effective before the formation of the synthetase:tRNA complex. Bluementhal et al (1973) reported a similar finding from their studies using E. coli RNA polymerase. In the second set of experiments, the concentration effect of the individual reaction components on the level of inhibition of a fixed concentration of ATA was examined. If the observed inhibition was due to the interaction of ATA with a certain reaction component, then raising its concentration should serve to overcome the inhibition. Of the four reaction components examined in this experiment (Mg^{++}, ATP, tRNA, and the synthetase), only alteration of the enzyme concentration had an effect on the level of inhibition by ATA. Reducing the enzyme concentration from 100 μg to 50 μg resulted in an increase in inhibition from 50% to 90%, while increasing the enzyme concentration to 150 μg resulted in essentially no inhibition. Therefore we conclude that the site of ATA interaction is the enzyme. The results from the third set of experiments confirmed this conclusion. Here, mixtures of partially purified enzyme and ATA as well as of tRNA and ATA were processed by a gel exclusion chromatography method to test for stable complex formation between ATA and reaction components. The results showed that stable complex formation could be demonstrated between ATA and the synthetase but not between ATA and tRNA. At the time these studies were conducted, there was no direct evidence for stable complex formation between enzyme and ATA to result in the observed inhibition, although there were some reports that ATA would bind to proteins (Lindenbaum and Schubert 1956).

We went further to study two basic questions: (1) what is the nature of ATA binding to the synthetase? and (2) how many ATA molecules can bind per enzyme molecule? These questions are the basis for the next step in the study of the mechanism of ATA action on the phenylalanyl-tRNA synthetase reaction.

In order to probe further into the nature of ATA binding to synthetase, an experiment was carried out to determine whether or not the bound ATA would interfere with the binding of various ligands to the enzyme. Binding studies using Millipore filtration and radioactively labelled precursors demonstrated that the presence of ATA at a concentration of 30 μM reduced the binding of tRNA to the enzyme to 30%. Binding of ATA and phenylalanine to the enzyme could not be demonstrated under these conditions and so the effect on these ligands remains for further study.

With the information that ATA binds to phenylalanyl-tRNA synthetase so as to interfere with the binding of other ligands, an attempt was made to obtain the stoichiometry of the ATA-enzyme interaction. For this purpose, a molecular weight determination of the partially purified synthetase had to be done. The enzyme migrated as a single peak on Sepharose 6B chromatography as detected by synthetase activity. Thus, knowing the relative position of marker proteins of known molecular weights, the molecular weight of the enzyme could be determined. The results show that the molecular weight of the synthetase is 70,000 daltons. The molecular weights reported for purified phenylalanyl-tRNA synthetase by other laboratories range from 150,000 to 280,000 daltons with a four subunit structure ($\alpha_2\beta_2$). Fasiolo (1973) reported a molecular weight of 220,000

with subunits of 56,000 and 63,000 for yeast phenylalanyl-tRNA synthetase while Weinstein et al (1973) reported a molecular weight of 280,000 with subunits of 69,000 and 74,000 for the same enzyme in rat liver. Purification of this enzyme in our laboratory subsequent to this thesis work established a molecular weight of 275,000 with subunits of 58,000 and 75,000 (Igarashi 1974). From these findings, it would appear that the above figure of 70,000 daltons for the synthetase is in the range of subunits rather than the intact enzyme and yet its position was established by assaying the fractions eluted from the Sepharose 6B column. The possible explanation for this discrepancy is as follows: if the conditions of the partial purification of the enzyme were such that the synthetase was dissociated into its subunits, then a mixture of the two types of subunits must have been subjected to gel exclusion chromatography. The Sepharose 6B column used in these experiments is not capable of clearly separating the mixture of proteins differing by less than 50,000 daltons in molecular weight, although it allows determination of peak positions of pure proteins processed separately. The result of the chromatography of the mixed subunits of molecular weight 75,000 and 58,000 would result in only a partial separation of the subunits with considerable overlapping at the region equivalent to 70,000 daltons--the molecular weight determined for the synthetase. Thus, the tube containing this overlapped region contains both subunits, so that reconstitution of an active form of the enzyme can occur in this particular fraction. Leaving the detailed study of enzyme structure to a further study, the number of ATA molecules bound per synthetase molecule was estimated assuming the molecular weight of the enzyme is

70,000 daltons. For this purpose, the fraction from the Sepharose 6B column chromatography containing the ATA:enzyme complex was used. The ratio of ATA to the enzyme in this complex was determined by their extinction coefficients at 308 nm and 280 nm respectively. The results show that there are 6 ATA molecules bound per enzyme of 70,000 daltons. This estimated value will be modified somewhat due to lack of critical information regarding the extinction coefficient of bound ATA. The extinction coefficients used here were for a typical protein solution (1 A_{280}/mg/ml/10 mm light path) and for a free solution of ATA. Though the value for the number of ATA molecules bound per synthetase is not likely the correct value, it does indicate that there is a limited number of sites on the enzyme where ATA can bind.

The results presented above remain somewhat qualitative in nature. However, the finding that aminoacyl-tRNA synthetase is sensitive to ATA was a novel one, and led to further detailed studies of the kinetic features of ATA action in this laboratory. In this respect, it is noteworthy that the kinetic analysis shows that ATA is a non-competative inhibitor at least for tRNA. This is a new finding and different from our original idea that the phenyl group with a suitable side chain may compete with the phenylalanine binding site. Obviously, the enzyme conformation changes upon binding with ATA and the binding sites for some of the ligands become inacessible. Further studies on the conformational changes of the enzyme is anticipated.

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